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THE EFFECTS OF DIET FORMULATIONS CONTAINING PROTEINS FROM DIFFERENT SOURCES ON THE INTESTINAL COLONIZATION OF *CAMPYLOBACTER JEJUNI* IN BROILER CHICKENS

A Thesis

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in Partial Fulfilment of the Requirements

of the Degree of

Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

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ABSTRACT

Campylobacter jejuni subsp. *jejuni* (referred to as *C. jejuni*) has emerged as the leading cause of foodborne bacterial gastroenteritis in humans world over, with an annual incidence of >2 million cases in North America alone. At times, it leads to complications such as reactive arthritis and a post infective polyneuropathy called Guillain-Barre syndrome. Chickens have been implicated to contribute to about 50 to 70% of human cases. Poultry are asymptomatic carriers of these organisms in their gastrointestinal (GI) tract where a commensal relationship has evolved. The colonization of *Campylobacter* in the GI tract of birds is very complex involving the host, pathogen and influenced by environmental factors. A good knowledge of the environmental factors that influence colonization is required to design an appropriate intervention strategy to reduce the colonization of these organisms in the chicken. The main objective of this research was to compare the effects of three diet formulations comprising of different protein sources (animal origin, plant origin and a combination of both animal and plant) in the colonization of *Campylobacter jejuni* in the GI tract of broiler chickens. A freshly isolated strain of *C. jejuni* (biotype IV serotypes HS O: 21, O: 29 HL untypeable) from a broiler chicken was used for infecting 3 day old chicks which were free of *C. jejuni* by a single oral administration of 0.5 mL of an inoculum containing 10^8 colony forming units. The shedding pattern of the organisms was also studied. Quantitative culture technique was used to enumerate *C. jejuni* in ceca, jejuni and crops of the birds. The isolates recovered from the birds were characterized and confirmed as *C. jejuni* using standard methods. The isolates were biotyped and serotyped at a National reference laboratory. The antimicrobial sensitivity profile of the isolates were determined with 12 commonly used antibiotics by the disk diffusion method. The minimum inhibitory concentrations (MIC) of 8 antibiotics were determined using E- test. Flagellin gene typing, a molecular based method was also used for characterization. Colonization of organisms was highest in the ceca of birds, ~ 10^4 to 10^6 cfu/g. Colonization in jejuni ranged from 10^2 to 10^4 cfu/g. A significant interaction was observed between diet formulations and colonization of *C. jejuni* in organs quantitatively cultured. The ceca of birds in the plant protein based feed group showed a statistically significant reduction in colonization whereas the effects on jejuni and crops were nonsignificant. The reduction in cecal colonization appears to be of practical significance because the main source of carcass contamination is from the rupture of the ceca during mechanized processing of birds. *C. jejuni* were recovered from the crops of birds. The recovery rate of *C. jejuni* ranged between 10^2 to 10^3 cfu/g. Further studies are required to ascertain whether the presence of organisms in the crop is a true colonization or a contamination, because the chances for the rupture of crop during processing are as high as that of the ceca. A cyclical pattern of shedding was observed in this study. Characterization, by biotyping and serotyping with Penner- HS scheme and flagellin gene typing, showed that 95% of isolates recovered from the experimental birds were identical to the strain inoculated. The remaining 5% of the isolates showed variation in phenotype in serotyping and genotype in flagellin gene typing suggesting the possibility of genomic rearrangements of those isolates taking place in the GI tract of birds. Antimicrobial resistance testing with disk diffusion and E- test did not reveal any significant differences in antimicrobial susceptibility of isolates in the different feed groups.

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^{0}C	- degree Celcius
$	- dollars
L	- liter
m	- meter
µm	- micrometer
µg	- microgram
µL	- microliters
M	- molar
mM	- millimolar
mV	- millivolts
%	- percent
pM	- picomolar
CD_{50}	- Colonization dose 50%
cfu	- colony forming units
spp.	- species
ANOVA	- Analysis of variance
ATCC	- American Type Culture Collection
AVC	- Atlantic Veterinary College
a_w	- water activity
BA	- Blood Agar
BCFA	- Branched Chain Fatty Acid
BHI	- Brain Heart Infusion
BSA	- Bovine serum albumen
CBF	- Campylobacter Blood Free selective medium
CCAC	- Canadian Council on Animal Care
CDC	- Centers for Disease Control and Prevention
CDT	- Cytolethal distending toxin
CE	- Competitive exclusion
C. jejuni	- *Campylobacter jejuni*
CHO	- Chinese hamster ovary
DFM	- Direct fed microbials
DM	- Dry matter
DNA	- Deoxyribo nucleic acid
EB	- Enrichment Broth
Eh	- oxidation reduction potential
EPEC	- Enteropathogenic *Escherichia coli*
FAE	- Follicle associated epithelial cells
FIL	- Feed induced lectin
FOS	- Fructooligosaccharide
GALT	- Gut associated lymphoid tissue
GBS	- Guillain-Barre syndrome

G + C	- Guanosine plus Cytosine
GI tract	- Gastrointestinal tract
HL	- Heat labile
HS	- Heat stable
IL-1	- Interleukin- 1
IU	- International units
LCT	- Long chain triacylglycerol
LOS	- Lipooligosaccharide
LPS	- Lipopolysaccharide
MCE	- Mucosal competitive exclusion
MCT	- Medium chain triacylglycerol
MHA	- Mueller- Hinton Agar
MH-BA	- Mueller Hinton-Blood Agar
MIC	- Minimum inhibitory concentration
MSRV	- Modified Semisolid Rappaport Vassiliadis
NCBI	- National Center for Biotechnology Information
NCCLS	- National Committee on Clinical Laboratory Standards
NLEP	- National Laboratory for Enteric Pathogens
NSP	- Nonstarch polysaccharide
OD	- Optical density
OMP	- Outer membrane protein
OR	- Oxidation reduction
PCR	- Polymerase chain reaction
PUFA	- Polyunsaturated fatty acid
RFLP	- Restriction fragment length polymorphism
RNA	- Ribonucleic acid
SCFA	- Short chain fatty acids
SEM	- Standard error of mean
sIgA	- secretory Immunoglobulin A
TAE	- Tris acetate buffer
TBE	- Tris borate buffer
TGF-β	- Transforming growth factor- β
UK	- United Kingdom
UPEI	- University of Prince Edward Island
USA	- United States of America
USDA	- United States Department of Agriculture
USFDA	- Unites States Food and Drug Administration
V	- Volt
VFA	- Volatile fatty acids
ZOI	- Zone of inhibition

1. REVIEW OF LITERATURE

1.1. General background

1.1.1. Food borne pathogens

Microbial contamination of food with concomitant illness has emerged as a major food safety issue on a global scale. According to the United States Food and Drug Administration (USFDA), about 33 million cases of food related diseases and 9,000 deaths occur annually (USFDA, 1997). One of the reasons for the increase in food borne diseases could be the increase in the individuals in human population vulnerable to diseases because of their age or health status. These infections are particularly a concern for the aged, infants and immunocompromised individuals. Other reasons could be changes in food handling procedures and consumption habits of the public. Contamination may occur at various stages of production, processing, distribution, marketing and handling of food. The Centers for Disease Control and Prevention (CDC) under USFDA has established certain pathogens as sentinel organisms to monitor for food borne infections because of prevalence, rapid multiplication, ease of transmission and fundamental difficulty in control containment.

The key organisms identified in food borne infections are *Campylobacter jejuni*, *Salmonella* spp., *Yersinia enterolitica*, *Listeria monocytogenes*, *Shigella* spp., *Vibrio* spp., and *Escherichia coli* O157: H7. *Campylobacter jejuni* and *Salmonella* spp. top the inventory of food borne bacterial infections in North America. Recently, concerns about certain microorganisms such as *Salmonella* and *Escherichia coli* (*E. coli*) that contaminate food are increasing because of the emergence of multidrug resistant *Salmonella typhimurium* definitive type 104 (DT- 104) and *E. coli* that have increased intestinal tissue adherence

properties and ability to secrete shiga like toxins (*E. Coli* O157: H7). *Campylobacter jejuni* has also become a concern as a diarrheal pathogen originating from food and water. Achieving the ultimate goal of food safety requires multifocal planning and co-operation between various agencies and individuals extending from the farm to the consumer.

1.1.2. Campylobacteriosis

Campylobacteriosis is caused by organisms belonging to *Campylobacter jejuni/ coli/ lari* species, and it has emerged as the leading cause of bacterial enteric disease in North America. It is estimated that about 2.4 million cases occur annually in the United States of America (USA) (Friedman et al., 2000), which is more than that of *Salmonella* and *Shigella* combined.

Campylobacteriosis is a zoonotic disease and infections in humans are chiefly food borne and foods of animal origin play a significant role in the transmission of the disease. Epidemiological studies have revealed a major association between *Campylobacter* infection in humans and handling and consumption of raw or undercooked poultry meat (Deming et al., 1987; Ooestrom et al., 1984).

1.1.3. Economic statistics at a glance

It has been estimated that the costs of the acute illness from food borne campylobacter are $ 471.7 million and the costs of campylobacter associated Guillian-Barre Syndrome (GBS) are $358.8 million. Combining acute and chronic costs, it causes an estimated $1.2 billion loss to the USA every year. This estimate does not include other costs involved such as travel to obtain medical care, time lost from work, costs of other chronic complications and pain and suffering (ERS USDA, 2001).

1.1.4. Times past

Campylobacter organisms have been recognized as animal pathogens since the 1900's. Investigations of abortions in sheep (McFadeyan, 1913) and cattle (Smith and Taylor, 1919) led to the discovery of a new, unidentified organism which was called *Vibrio fetus*. In 1931, a vibrio was isolated from the jejunal mucosa of cattle with dysentery and also from calves with enteritis. This organism was similar to *Vibrio fetus* but antigenically distinct and so was called *Vibrio jejuni*. Doyle (1944) isolated a vibrio from feces of pigs with diarrhea and named it *Vibrio coli*. King (1957) observed these organisms in the blood of humans with diarrhea. In 1963, the name of the organism was changed to *Campylobacter jejuni* (Sebald and Veron, 1963).

1.1.5. Microbiology

Campylobacter are gram negative, slender, curved and motile rods measuring about 0.5 to 8.0 μm long and 0.2 to 0.5 μm wide (Penner, 1988). The genus *Campylobacter* is placed in the delta/ epsilon subdivision of *Proteobacteria* which includes *Helicobacter* and *Arcobacter* (NCBI, 2002). The genus name *Campylobacter* comes from Greek which means a curved rod (Sebald and Veron, 1963). They are microaerophilic requiring reduced levels of oxygen for growth. Sebald and Veron (1963) proposed to include microaerophilic bacteria and other vibrios that were unrelated to *Vibrio cholerae* on the basis of their nucleotide base composition (mol% Guanine plus Cytosine) as well as their ability to use sugars either oxidatively or fermentatively, into another genus, *Campylobacter*. Their guanine plus cytosine (G + C) content is low, about 28 to 38 mol% (Smibert, 1984). Although *Campylobacter* organisms are microaerophilic, their metabolism is aerobic and

utilize amino acids and intermediates of the tricarboxylic acid cycle. Campylobacters are unable to ferment or oxidize carbohydrate substrates. The *Campylobacter* spp. of medical importance such as *C. jejuni, C. coli, C. lari* are thermophilic and require 42°-43° C for optimal growth, unlike *C. fetus* which prefers 25° C. This feature is used to distinguish thermophilic campylobacters from other campylobacters. About 95-98% of campylobacter infections in humans are caused by *C. jejuni* while the rest are caused by *C. coli* (Karmali et al., 1983). On primary isolation campylobacters exhibit two colony forms, (1) gray, flat, irregular and spreading type especially on freshly prepared media and (2) round, convex and glistening with little spreading (Smibert, 1978). The growth of the organism is fastidious, requiring about 24-48 hours for recognizable growth, but 48-72 hours when only very few organisms are present in the inoculum.

Isolation of campylobacters requires specialized laboratory techniques. Suitable media for transport and storage include a semisolid brucella medium with 10% sheep blood (Wang et al., 1980), Cary Blair media with decreased agar (Cary and Blair, 1964), and alkaline peptone water with thioglycolate and cystine (Wang et al., 1983). Selective media that support the growth of campylobacters and at the same time suppress the growth of other enteric microbes are necessary for their isolation. Many different formulations have been used for selective isolation of campylobacters. Selective media include blood free media such as charcoal cefoperazone deoxycholate agar (CCDA) (Hutchinson and Bolton, 1984), charcoal based selective medium (CSM) (Karmali et al., 1986) and semisolid blood free motility medium (SSM) (Goosens et al., 1989), and blood containing media such as Campy-CVA medium (Reller et al., 1983) and Skirrow medium (Skirrow, 1977). Preston blood free

medium which contains cefoperazone works well for routine isolation of thermophilic campylobacters (Bolton and Coates, 1983).

1.1.6. Identification

The identification of campylobacters poses certain difficulties because of their fastidious nature of growth and inability to ferment sugars. The standard tests employed for characterizing campylobacters are oxidase, catalase, hippurate hydrolysis, latex agglutination and susceptibility to antibiotics, particularly nalidixic acid and cephalothin. *C. jejuni* is positive for oxidase, catalase, latex agglutination and hippurate tests, grows at 42^0 C, and is sensitive to nalidixic acid but resistant to cephalothin. However, susceptibility to nalidixic acid can no longer be regarded as a reliable means of differentiating *C. jejuni* and *C. coli* from other members of the genus *Campylobacter*. Nalidixic acid resistant campylobacters may be misidentified as *C. lari* or other species that are intrinsically resistant to this compound (Altwegg et al., 1987). The distinction between *C. jejuni* and *C. coli* is entirely dependent on a single test, the hippurate hydrolysis. *C. jejuni* is hippurate positive while *C. coli* is hippurate negative, although hippurate negative *C. jejuni* and hippurate positive *C. coli* have been observed (Goossens and Butzler, 1992).

1.1.7. Serotyping of *Campylobacter jejuni*

Subtyping of *Campylobacter jejuni* is essential for epidemiological purposes to discriminate between strains reliably and accurately in order to elucidate the sources and modes of transmission. Several methods have been described for typing *Campylobacter* spp. including biotyping, serotyping, plasmid typing, enzyme profile analysis, restriction digest analysis, and phage typing. Molecular methods such as restriction endonuclease analysis

(Patton et al., 1991), analysis with genes encoding for rRNA (Kiehlbauch et al., 1991), plasmid analysis (Bradbury et al., 1983), multilocus enzyme electrophoresis analysis typing (Aeschbacher and Piffaretti, 1989) and flagellin gene typing (Nachamkin et al., 1996) have been used in the recent years to characterize campylobacters. However, serotyping to detect heat labile (HL) antigens or O (heat stable) antigens has played an important role in understanding the epidemiology and identifying the source of campylobacter infections.

Serotyping schemes developed in Canada, based on heat stable (HS- Penner) and heat labile (HL- Lior) antigens are the most sensitive and discriminatory phenotypic methods for typing *Campylobacter jejuni* and have gained international acceptance as reference methods (Penner and Hennessy, 1980 and Lior et al., 1982). These methods have played an important role in epidemiological investigations and identifying the source of infection of campylobacter organisms (Patton et al., 1991).

Biotyping is also used commonly, and consists of tests that determine resistance to certain antibiotics, dyes, and chemicals. On the basis of test arrangements and results, a figure code indicates both species and biotype (Patton and Wachsmuth, 1992).

To standardize serotyping on an international basis, the International Committee on Serotyping *Campylobacter* (1985) recommended that the systems of Penner and Lior be adopted and referred to as the HS and HL systems respectively (Patton and Wachsmuth, 1992). The Penner scheme has been used extensively and forms the basis for a scheme (LEP scheme) recently developed at the Laboratory of Enteric Pathogens, Public Health Laboratory Services, UK (Frost et al., 1998). The HS antigen extract, instead of viable subculture can be used for serotyping with HS system whereas, HL system needs live cells

(Fricker, 1986).

Although serotyping schemes have good discriminatory potential in identifying unrelated strains, they are complex and laborious and require reagents that are expensive and time consuming to produce. Hence, very few laboratories provide serotyping facilities and they are therefore not easily accessible to researchers (Patton and Wachsmuth, 1992). The level of untypeability is a concern in both serotyping schemes, particularly the HL scheme. In general, nontypeability of the HS scheme in human and veterinary strains is reported to be less than 20%. Jacobs-Reitsma et al. (1995) reported a rate of 20% untypeability while Neilson and Neilson (1999) stated that 16% of strains of poultry origin were untypeable. According to Salama et al. (1990) and Jacobs-Reitsma et al. (1995) the typeability of strains was clearly affected by storage of strains before typing. Yan et al. (1991) pointed out that the influence of environmental factors on the stability of most of these phenotypic properties on which the serotyping schemes rely on is a major weakness of these techniques.

1.1.8. Pathogenic properties

Campylobacter jejuni have been identified as a significant cause of enteric disease in humans and animals. The first account of its pathogenic property in humans comes from King (1957) who isolated this organism from the blood of children with diarrhea. The exact mechanism by which campylobacters produce disease has not been fully elucidated. However, three mechanisms have been postulated such as (i) adherence and production of enterotoxins, causing secretory diarrhea (ii) invasion and proliferation within the intestinal epithelium causing damage and an inflammatory response clinically manifested as dysentery and diarrhea and (iii) translocation in which the organisms penetrate the intestinal mucosa,

causing minimal damage and proliferation in the lamina propria and mesenteric lymph nodes, with occasional extraintestinal infections such as meningitis, cholecystitis, urinary infections (Levine et al., 1983).

The infective dose is very small and human feeding studies suggest that only about 400-500 bacteria are needed in some individuals to cause disease (Black et al., 1988). Rarely, complications are observed such as reactive arthritis, hemolytic uremia syndrome and septicemic infections of any organ. Fatalities are rare in healthy individuals but may occur in immunocompromised or debilitated patients. Meningitis, recurrent cholecystitis, and post infective polyneuropathies such as Guillian-Barre syndrome and Miller-Fisher syndrome occur as very rare complications (Allos, 1997; Altekruse et al., 1999).

Introduction of pathogenic strains derived from humans with enterocolitis induced diarrhea in newly hatched chicks (Ruiz-Palacios et al., 1981). Approximately half of the strains isolated from human diarrhea cases produced a cholera related enterotoxin (Hariharan and Panigrahi, 1990). Certain strains of *C. jejuni* from human cases of GBS have been shown to cause paralysis in young chicks (Hariharan et al., 1999).

1.1.9. Epidemiology

Most animals and poultry, domesticated as well as wild, are asymptomatic carriers of campylobacter organisms in their gastrointestinal (GI) tracts (Skirrow, 1982). Campylobacters are ubiquitous organisms and their epidemiology is quite complex. The organisms have been isolated from the GI tracts of cattle, pigs, sheep, dogs and cats, feral animals and birds. Wild birds also carry these organisms in their GI tracts.

However, the greatest significance of these organisms is attributed to the intestinal

carriage in domesticated broiler chickens. This is due to the potential for contamination of carcasses during mass mechanized processing. In North America, most of the chicken marketed are contaminated with *C. jejuni.* A recent two year study by the Minnesota Department of Health found that 88% of poultry sampled from local supermarkets tested positive for campylobacters (USFDA, 2000). Processing and packaging of chicken meat provides suitable conditions that favor survival of campylobacters (Fricker and Park, 1989). There is evidence that campylobacters survive better on poultry meat than on pork carcasses (Ooestrom and de Wilde, 1983). Poultry meat tends to be moist when sold and this moisture may protect campylobacters during storage (Fricker and Park, 1989). Feces from infected birds may contaminate the surface of eggs.

A seasonal pattern has been observed in the incidence of campylobacter infections with most cases occurring between the summer months of July to September followed by a second peak extending from mid November through mid December. Non-chlorinated water and unpasteurized milk also have been implicated in outbreaks although their contribution is comparatively low. Most of the human infections are due to consumption of uncooked or under cooked poultry meat. Vegetables could become a potential source of the infection as Kumar et al. (2001) have reported isolation of *Campylobacter jejuni* biotype 1 from 2 vegetable samples out of 56 screened. Many studies point to the fact that most human infections are derived from chicken-origin strains. A significant correlation exists between handling or preparing of raw chicken and occurrence of confirmed *C. jejuni* infections (Hopkins and Scott, 1983). Free ranging chickens kept under backyard systems of management in which humans have a close contact with birds can serve as a potential source

of infection (Marquis et al., 1990). Workers in poultry processing plants and abattoirs are also more likely to get infections (Grados et al., 1983).

1.1.10. *Campylobacter* carriage in poultry

Most domesticated and wild birds are natural reservoirs of campylobacters. Pheasants, quails, gulls, puffins and shore birds such as waders are frequently infected with campylobacter organisms (Cabrita et al., 1992). In a study conducted in Prince Edward Island, the majority of broiler chickens were found to harbor *C. jejuni* in their intestinal tracts (Ahmed et al., 1992).

1.1.11. Transmission

Campylobacters are ubiquitous in the environment surrounding the broiler house and without doubt environmental contamination is the greatest potential source of infection in a new flock. Recent studies by Cox et al. (1999) and Pearson et al. (1996) suggest that vertical transmission through contaminated eggs may play a role in the transmission of *C. jejuni.* At first a few birds are infected and these birds are known as seeder birds. Seeder birds can rapidly spread the organism to a majority of hatch mates within three days or less (Clark and Bueschkens, 1986; Shreeve et al., 2000). Because of the coprophagic habits of birds, fecal oral spread is possible. There occurs a transient palatine colonization of *C. jejuni* in young chickens. Since chickens drink water from a common water source, *C. jejuni* could be transmitted to as many as 20,000 birds in a logarithmic progression (Montrose et al., 1985). This hypothesis was supported by a number of studies (Smitherman et al., 1984; Engvall et al., 1986; Pokamunski et al., 1986).

Annan-Prah and Janc (1988) monitored 10 broiler flocks but did not isolate the organism from their samples until the birds were 21 days old. They suggested that other farm animals and farmers' boots could have been sources of contamination of flocks. Lindblom et al. (1986) indicated that once *C. jejuni* appeared in a flock, it spread rapidly to virtually all birds. Stern et al.(1988) reported that the dose required to colonize 50% of orally challenged 24 hour old chickens (CD_{50}) was only 35 colony forming units (cfu).The reason for not typically isolating the organism from the hatchery environment, or during early stages of production is that the organisms invade the production facilities sporadically. As soon as this invasion does occur, the entire flock of chicken is at risk. Specific Pathogen Free chicks, when placed on contaminated litter, started fecal shedding within 5 days, and it persisted for 46 days (Montrose et al., 1985). The organism can remain viable in the litter for some period (perhaps as long as 2 weeks), but becomes inactivated fairly rapidly (Montrose et al., 1985). Achen et al. (1998) have observed a cyclicity in fecal shedding of *C. jejuni* in individual birds.

The reduction of *C. jejuni* in the GI tract of chickens has become a major focus of research. Several strategies, such as production level control measures, genetic variation of chickens to campylobacter colonization, development of vaccines, have been attempted. However, the general consensus is that the most effective strategy would be intervention measures directed at farm level production control. This task is formidable because campylobacters are ecologically adapted to colonize the avian intestinal tract without producing any disturbances or ill effects on their hosts.

1.2. Virulence determinants and microenvironmental factors that influence the colonization of *Campylobacter jejuni*

1.2.1. Background

The interaction between pathogens and their hosts in the infectious process is very complex, involving the host, the organism and environmental factors. The hosts have developed several mechanisms to defend themselves from the invasion of pathogenic organisms. At the same time organisms have also evolved several subtle mechanisms to avoid or even subvert the host defense mechanisms.

A good orchestration of many sequential events in the pathogenic processes is needed depending on the environmental cues encountered in the host by appropriate expression of virulence factors. The environmental cues involved are changes in temperature, pH, osmotic pressure, and availability of oxygen and nutrients. Virulence of pathogens, i.e., their ability to produce morbidity and mortality in a host, in general is an extremely complex and multifactorial process requiring the coordinated activity of many bacterial gene products and involving several other components such as motility, adherence, iron acquisition ability and enterotoxin production.

Microorganisms can express alternative cellular adherence mechanisms depending on the environmental conditions encountered on different host surfaces. In addition to colonization of the mucus layer and adherence to the mucosal cell surface, invasive bacteria appear to bind transiently to the host cells prior to internalization (Chiang et al., 1999). Little is known about what adherence functions are important in colonization and why chickens are susceptible to colonization but at the same time not to disease. Studies of bacterial

responses to changes in pH, temperature and iron levels and analysis of host cell invasion and survival in macrophages have all been used to identify and characterize bacterial virulence determinants. Although in vitro screens have been useful to provide much information on the mechanisms of bacterial pathogenesis, they will not accurately reproduce all aspects of the host pathogen interaction. In vivo experimental models are highly desirable but have limitations in that they are very expensive and labor intensive.

1.2.2. Indigenous microflora

The indigenous flora of an animal, according to the definition given by Dubos et al. (1965) are those composed of the autochthonous microorganisms that natively colonize a particular habitat or environmental niche and the allochthonous flora as microorganisms that cannot colonize a particular habitat except under abnormal circumstances. Autochthonous microbiota were present during the evolution of an animal and therefore, present in all communities of a particular animal species. Allochthonous organisms are acquired transiently and therefore, are not necessarily present in all communities or even present in all members of a single community of animals.

Experiments in mice have demonstrated that secretory immunoglobin A (sIgA) antibodies from breast milk affect the normal GI flora in the suckling newborn by retarding its contact with the developing Gut Associated Lymphoid Tissue (GALT) (Kramer and Cebra, 1995). The presence of Transforming Growth Factor- β (TGF- β) might further contribute to its tolerogenic properties because this cytokine exerts a pronounced immunosuppressive effect on GALT and it may play a part in isotype switch to IgA (Ishizaka et al., 1994) and it supports the epithelial barrier function (Planchon et al., 1994). When the

host's immune response is successfully elicited, GALT will be further protected by the sIgA antibodies produced in the GI tract; local immunostimulation is thereby attenuated despite the continued presence of microorganisms (Shroff et al., 1995). This could partly explain the hyporesponsiveness that exists toward members of the indigenous gut flora resembling the oral tolerance observed both in rodents and in humans (MacDonald, 1995).

1.2.3. Microbial colonization in the GI tract

The term colonization is often designated as the formation of a stable population of bacteria in a suitable habitat (Arp, 1988). The GI tract is a complex ecosystem harboring a diverse and highly evolved microbial community composed of hundreds of different microbial species (Dunne, 2001). The indigenous flora of the human GI tract comprises a remarkably complex and stable colony of more than 400 separate species, living in a symbiotic relationship with the host. Chickens also harbor hundreds of different species of indigenous organisms in their GI tract (Rolfe, 1991). The indigenous flora influences multiple aspects of physiologic homeostasis and forms a key component of normal host defenses against infection by exogenous pathogens (Marshall, 1999). The organisms have acquired a wide range of sophisticated adaptations which allow them to survive in the many ecological niches provided by the gut surface during the evolutionary process (Lee et al., 1986).

Some indigenous microbes form climax communities on gastrointestinal epithelial surfaces during succession in animals of many types (Savage, 1978). Because of the complex of continuing interactions between environmental factors and microorganisms, food at any

one point carries a characteristic flora called its association. The microbial profile changes continuously and one association succeeds another in what is called a succession (Sinell, 1980). One group of organisms after another become dominant only later to be suppressed by organisms which are in turn suppressed (Rolfe, 1991).

Most bacterial communities are formed in mucous layers on the surfaces, where some microbes in the communities may utilize the mucin as a source of carbon and energy. The mucous gel may also serve physically to stabilize the community. Some microbial types that colonize early during succession may serve, perhaps synergistically with dietary components, to lower the oxygen tension and oxidation reduction potential so that oxygen intolerant anaerobes can colonize the epithelial surfaces. Dietary fiber may influence the composition of these communities by providing nutrients for the microbes or by altering their environmental conditions including peristaltic rate, mucous concentration, composition, oxygen tension and oxidation-reduction potential (Savage, 1978).

1.2.4. Mechanisms used by pathogens to colonize and penetrate mucosa

1.2.4.1. Mucus penetration

Mucosal surfaces have a thick covering of mucus which forms a key component of the first line of defense against pathogens. Mucus is composed of glycoconjugates secreted by specialized exocrine goblet cells. They form viscous gels that trap microorganisms and limit their diffusion to the epithelium. The interaction between microbes and mucin involve mucin carbohydrate side chains and microbial adhesion molecules. Certain microorganisms may alter mucin biochemistry or expression, which may contribute to disease production (Bailey et al., 1993).

Motility, chemotaxis and adhesion play key roles in microbial ecology and appear to promote colonization by allowing certain organisms to selectively seek out nutrients or sites of colonization (Kennedy, 1987). Motility appears to be involved in *V. cholerae* colonization (Freter et al., 1981). In the case of *Salmonella* spp. entry mechanisms are quite complex, involving intimate interaction between the bacterium and the host cell resulting in a cross talk in which biochemical signals are presumably exchanged. As a consequence, a complex set of signaling events are triggered in the host cell leading to marked cytoskeletal rearrangements, membrane ruffling and bacterial uptake by macropinocytosis. Surface determinants such as flagella or lipopolysaccharides (LPSs) may help the entry process by facilitating contact of the bacteria with the host cell (Galan, 1996). Organisms such as *Shigella* and *Yersinia,* which are nonmotile, are capable of penetrating mucosal surfaces and interacting intimately with mucosal epithelial cells.

Certain organisms which lack several virulence components are still successful colonizers as they show exceptionally high competence in relation to others. For example, *E. coli* K88 need not be motile to be pathogenic but shows strong adhesion to epithelial cells (Freter, 1981). Some bacterial toxins that lead to diarrhea also lead to the loss of mucus and this loss may facilitate access to mucosal epithelial cells (Siebers and Finlay, 1995).

1.2.4.2. Adherence

The initial step toward colonization and establishment of infection is the adherence of the bacterium to the host cell. It requires the participation of two components; an adhesin of bacteria and a receptor on the host cell surface. The mechanism used for adherence usually involves binding of bacterial surface appendages such as pili (fimbriae)

to host cell surface receptors (Ghose, 1996). In the case of virulent strains of *E. coli,* specific adhesins including P fimbriae and type 1 fimbriae appear to aid in colonization and virulence (Winberg et al, 1995). Bacteria can make nonfimbrial adhesins that can mediate adherence. Lipooligosaccharides (LOS) and LPSs of gram negative bacteria serve a critical role as the principal interface between the organism and environment. LPS/ LOS are involved in the adherence of *Aeromonas sobria, Campylobacter jejuni, E. coli, Helicobacter pylori, Salmonella typhi* and *Serratia flexneri* (Jacques, 1996). Merino et al. (1996) found that the O antigen LPS is important for the colonization of *Aeromonas hydrophila* serogroup O: 34 in germ free chickens.

A gastric lipid, phosphatidylethanolamine (PE) has been described as a receptor for *Helicobacter pylori* (Lingwood et al., 1992). Urease has been found to be vital for the colonization and pathogenesis of *Helicobacter pylori* (Ohta et al., 2001). In addition to adherence to the mucosal surface receptors, many bacterial adhesins mediate bacterium-bacterium contact, resulting in formation of apically bound microcolonies. Some pathogens that mediate this type of adherance include enteropathogenic *E. coli* (EPEC) and *V. cholerae* (Irdell and Manning, 1994).

1.2.4.3. Epithelial cells

The apical surface of intestinal epithelial cells have microvilli to which mucosal pathogens adhere. Some pathogens adhere to microvilli at their tips and do not distort the host cell surface. However, many pathogens such as *Salmonella* alter the apical surfaces often denuding the microvilli and there by providing additional surface area to interact (Finlay and Falcow, 1990). Several mucosal pathogens such as *Yersinia* and *Shigella*

although unable to adhere to the apical surfaces of polarized epithelial monolayers; interact specifically with basolateral surfaces (Mounier et al., 1992). This specificity is enigmatic as basolateral surfaces are not exposed to incoming pathogens. One way of circumventing this, is to use the M cells which indiscriminately take up many foreign materials. Another mechanism is used by *Shigella* spp. which trigger chemotaxis of phagocytic cells (polymorphonuclear leukocytes). When these cells move toward bacteria, they span the mucosal epithelial barrier and open the tight junctions, and at that time *Shigella* are either internalized by these cells or they slip through the intercellular route to the basal area of the mucosa (Perdomo, 1994).

1.2.4.4. Invasion

Many pathogenic bacteria possess the capacity to enter nonphagocytic eukaryotic cells. The mechanisms used by these bacteria vary between organisms, and as does the role of invasion in pathogenesis (Tang et al., 1993). Most invasive organisms appear to exploit existing host signal transduction systems to facilitate a cytoskeletal rearrangement that mediates bacterial uptake. The capacity to enter and then penetrate through (transcytose) epithelial barriers facilitates bacterial breaching of epithelial barriers.

Both *Shigella* and *Yersinia* can invade cells, yet neither can invade the apical surface of a polarized cell. It is thought that they penetrate through M cells, thereby gaining access to the basolateral surfaces of epithelial cells that they then invade. Possibly *Shigella* spp. also penetrate through or next to polymorphonuclear leukocytes spanning the epithelial barrier in the process of transmigration (Perdomo et al., 1994). Secreted proteins are a key component in the pathogenesis of a variety of bacteria such as *Salmonella*, *Shigella*, *Yersinia*

and enteropathogenic and enterohemorrhagic *E. coli*. Enteropathogenic *E. coli* secrete proteins that elicit cytoskeletal rearrangements on target host cells (Mobley, 2000).

M cells or follicle associated epithelial cells (FAE) are specialized epithelial cells over the surface of lymphoid follicles which have microfolds over their luminal surface instead of microvilli (Owen, 1999). It appears that most organisms penetrate through M cells, and the lack of mucus barrier on M cells suggests that mucus probably does not play a significant role in the colonization of these specialized cells.

Many foreign materials are taken up by the antigen delivery system of M cells: macromolecules (horseradish peroxidase, ferritin, lectin), viruses (Reo, Polio, Human Immunodeficiency Virus), bacteria and protozoa (Keren, 1992). This defense system is subverted by some pathogens to make entry into the hosts. They utilize the transepithelial movement through M cells as an invasion route. *V. cholerae* is a noninvasive enteropathogen that colonizes the mucosal surface of the small intestine (both villi and follicles) whose uptake across the epithelium is specifically performed by M cells. Exocytosis into M cell pockets results in dissemination of free or macrophage engulfed bacteria into the follicle dome (Owen et al., 1986). Certain genetic microbial characteristics control attachment of *Salmonella* to M cells and induce M cell ruffling and bacterial uptake (Jones et al, 1994). Invasive pathogens also use M cells as the main portal of entry across the epithelial barrier. Such pathogens include *C. jejuni* (Walker et al, 1988), *Salmonella* (Jones et al., 1994), and *Shigella flexneri* (Wassef et al., 1989).

1.2.4.5. Toxins

An important component of bacterial infection of the GI tract, particularly when enterotoxin disruption of gut function occurs, relates to the interaction of the bacterium and toxin with the intestinal surface. Adherence or attachment of bacteria lead to colonization and toxic or invasive states (Walker, 1986). Cholera toxin penetrates epithelial cells and alters ion transport but does not disrupt intercellular junctions. *V. cholerae* also secretes a toxin (Zot) that specifically disrupts tight junctions and this can contribute to the production of diarrhea (Fasano et al., 1991). Virulent strains of *E. coli* produce several toxins such as the pore forming hemolysin, cytotoxic necrotizing factor and an autotransported protease, called secreted autotransported toxin (Sat) (Mobley, 2000).

1.2.5. Colonization of *Campylobacter*

Campylobacter jejuni can be considered as a very successful organism for the following reasons: (i) It can colonize the GI tract of birds without eliciting immune responses in the host (ii) It has evolved several strategies for long term survival in several host species and (iii) It has the ability to produce disease in susceptible species with a limited number of organisms. Oral inoculation studies have revealed that *C. jejuni* can readily colonize chicks (Beery et al., 1988). Several factors influence the organism's ability to colonize chicks, including the number of cells and strain of *C. jejuni,* source, cultural history, and the lineage and age of chicks (Stas et al., 1999). Strains differ in their capacity to colonize the intestines in chickens (Meinersmann et al., 1991; Stas et al., 1999).

1.2.5.1. Sites of colonization

The ceca and cloaca are the principal sites of campylobacter colonization (Beery et

al., 1988). The organism was recovered most frequently from the ceca, distal small intestine, large intestine and cloaca (Beery et al., 1988). In the ceca the organism was often detected at levels of 10^4 to 10^7 cells/g (Beery et al., 1988). Studies by Di Modugno et al. (1997) have indicated that *C. jejuni* can colonize the oviducts of laying hens. These organisms might probably be migrating from the cloaca. Recently, several workers have reported isolation of campylobacter organisms from the crop of birds (Byrd et al., 1998, Achen et al., 1998). In humans the preferred sites are jejunum and ileum (Ruiz- Palaicos et al., 1981) and colon (Karmali and Fleming, 1979).

Histological study of the ceca and cloaca of chicks orally inoculated with *C. jejuni* revealed that they were localized in the lumen of mucus filled crypts (Doyle, 1991). *C. jejuni* appeared to be confined to the lumen of the crypts and there was no evidence of any pathological changes (Doyle, 1991). According to Doyle (1991) 4-35% of cecal crypts were filled with densely packed *C. jejuni* cells. *C. jejuni* generally filled the crypts from the proximal end to the distal end, but were most densely concentrated at the distal end (Doyle, 1991). Examination of cecal crypts colonized by *C. jejuni* by electron microscopy revealed that they pervaded the lumina crypts and were occasionally in close apposition to the glandular microvilli but were never in direct contact with the microvillus outer membrane (Doyle, 1991). It appeared that *C. jejuni* colonized crypt mucus without attaching to crypt microvilli (Doyle, 1991). Lee et al. (1986) observed that in mice, *C. jejuni* colonized mucus on the outer surface and deep within the intestinal crypts, with the cecal crypts being preferentially colonized. *C. jejuni* do not adhere to the intestinal surface but are highly motile and track rapidly along intestinal mucus (Lee et al., 1986).

Campylobacter jejuni preferentially colonize many parts of the gastrointestinal tract depending on microenvironmental factors such as oxygen tension, pH and presence of host receptors (Lee et al., 1983). However, there is very little information available on the effects of pH, temperature, oxygen tension, nutrients etc in vivo on the colonization of campylobacters.

1.2.5.2. Bacterial factors

Many pathogens have the ability to attach to nonprofessional phagocytic cells. This is considered to be an important virulence factor as it prevents the organism from being swept away by forces such as peristalsis and fluid flow (Isberg and Van Nhieu, 1994). Adherence is determined by specific interaction between molecules on host and bacterial surfaces. Adherent organisms are partially protected from host immune responses (Isberg and Van Nhieu, 1994). The colonization of mucosal surfaces by *C. jejuni* depends on the ability of the bacteria to maintain close proximity to and attachment to the mucosa and avoid being swept away by the movements of the intestinal contents (Griffith and Park, 1990).

1.2.5.3. Association with intestinal mucosa

The association of *C. jejuni* with intestinal mucosa in humans is facilitated by various factors such as the morphology of the bacterium, motility and chemotaxis (Walker et al., 1986). The spiral shape of the organism and its characteristic cork screw motility enable it to penetrate and pass through the thick intestinal mucus and thus establish a close contact with the intestinal mucosa (Griffith and Park, 1990).

C. jejuni is well adapted for mobility in viscous solutions such as mucus which may provide the organism with an ecological advantage in the gastrointestinal tract. The *C. jejuni* were seen to be highly motile in the intestinal tract, rapidly tracking along the intestinal mucus (Lee et al., 1983). The ability to colonize intestinal mucus is a major determinant of pathogenicity in intestinal *Campylobacter* infections. According to Walan and Kihlstrom (1988) all strains of *C. jejuni* were negatively charged and possessed a hydrophobic surface which could participate in adherence (Kervella et al., 1993). Freter et al. (1979) observed that active or passive penetration of mucous gel will have similar ecological functions as binding to epithelial cell surfaces and trapping in the mucous gel may even represent the only way in which bacteria associate with the mucosa.

Hugdahl et al. (1988) studied the chemotactic behavior of *C. jejuni* in the presence of different carbohydrates, amino acids and preparations and constituents of mucin and bile. Of the various carbohydrates studied, only L-fucose, a galactose analog, was found to be a chemoattractant. L-cysteine, L-glutamate, L-aspartate and L-serine were found to be chemotactic. Organic acids found to be chemotactic were pyruvate, succinate, fumarate, citrate, malate and α- ketoglutarate. McSweegan and Walker (1986) studied the role of carbohydrates in the adherence of *Campylobacter* species to epithelial cells and found that fucose and mannose were inhibitory but neither sugar completely inhibited adhesion. Rhamnose exhibited a concentration independent inhibitory effect (Naess et al., 1988). Szymanski and Armstrong (1996) found that *C. jejuni* was capable of binding to lipids and the binding was greatest to entire lipid structure but affinity decreased when portions of the lipids were removed.

Mucin, a glycoprotein of high molecular weight, is the principal constituent of mucus and was found to be a chemoattractant for *C. jejuni*. Mucins, excreted from epithelial cells of intestinal, gastric, and gallbladder tissue, have a similar structure, consisting of an extended protein core with attached oligosaccharides. The oligosaccharides attach to the protein core via serine and threonine ether linkages. The carbohydrate constituents are sialic acid, i. e., substituted neuraminic acid, or L- fucose or both attached to N- acetylglucosamine, N- acetylgalactosamine, and galactose. Mucin is principally composed of carbohydrate molecules and L- fucose is invariably a terminal sugar.

The protein core of mucin primarily contains serine, threonine and proline characteristically in large amounts. Chemotaxis toward serine or other amino acids components of mucin would be less likely than chemotaxis toward the peripheral L- fucose. Therefore, it is likely that the L- fucose moiety is the principal chemoattractant in mucin (Hugdahl et al., 1988). Oxgall, fresh chick bile, and fresh beef bile were determined to be chemoattractants: however, many of the individual components of bile were chemorepellants, including cholic acid and its derivatives. The only component of bile determined to be a chemoattractant was mucin. Mucin also serves as a sole substrate for growth of *C. jejuni*. Beery et al. (1988) hypothesized that *C. jejuni* is attracted to the mucin component of mucus, in which it moves by a highly active flagellum to mucin filled crypts where the organism establishes itself. *C. jejuni* probably grows by using mucin as a substrate within the crypts. The organism is likely to remain established in the crypt because of its chemoattraction and metabolism of mucin. *C. jejuni* does not appear to attach to the crypts. Adherence to the crypts appears not important for colonization of the ceca and cloaca. More

recently, Takata et al. (1992) confirmed that chemotactic movement is important for intestinal colonization.

1.2.5.4. Pili and OMPs

Pili or fimbriae and afimbrial adhesins are two important components involved in the attachment of *C. jejuni*. However, the ability to survive in the mucous by binding to mucous components itself can be considered a virulence factor, as mucous forms part of the defense mechanism of the body. Previously it was thought that *C. jejuni* did not produce fimbriae, yet a report by Doig et. al. (1996) indicated that synthesis of pili did occur in the presence of bile salt deoxycholate in several strains of *C. coli* and *C. jejuni*. However, recently, Gaynor et al. (2001) reported that these appendages are not pili but are a bacteria-independent morphological artifact of growth medium. Other possible adhesins include outer membrane proteins (OMPs), flagella and LPS. *C. jejuni* has also been reported to bind to extracellular matrix components such as fibronectin, laminin, vibronectin and collagen, but the specific roles played by these components in adherence is not fully understood (Konkel et al., 1993).

1.2.5.5. Flagella

The role of flagella in the colonization of *C. jejuni* has been studied in the GI tract of infant mice (Newell et al., 1985). They found that a flagellate, nonmotile variant colonized the GI tract as successfully as the wild type strain; however, the aflagellate variant poorly colonized the mouse. They concluded that flagella, whether active or inactive, are necessary for the efficient colonization of the GI tract of infant mice by *C. jejuni*. Using an experimental approach similar to the model used by Newell et al. (1985), Morooka et

al.(1985) confirmed the role of flagella in the colonization of GI tract. Flagellin has been suggested as an adhesin or carrier of adhesin since nonflagellated mutant strains adhere much less efficiently to host cells (McSweegan and Walker, 1986).

C. jejuni show enhanced motility in the mucous, which usually immobilizes peritrichously flagellated bacteria (Szymanski et al., 1995). They can move very actively in mucous filled crypts without any evidence of attachment to epithelial cells, which is essential for colonization in the intestinal tract (Szymanski et al., 1995). The flagellum of *C. jejuni* is composed of two closely related proteins, the major subunit Fla A and minor subunit Fla B encoded by *fla A* and *fla B* genes respectively (Guerry et al., 1991). The motility imparted by flagella aided by chemotactic signaling may allow *C. jejuni* to penetrate the mucous layer and seek host cell receptors involved in colonization or invasion of intestinal mucosa (Takata et al., 1992).

1.2.5.6. Toxins

C. jejuni produce enterotoxins, cytotoxins, hemolysins, and adherence factors (Wassenaar, 1997). *C. jejuni* strains may produce toxins related to *V. cholerae* and *E. coli* LT (Hariharan and Panigrahi, 1990; Ahmed et al., 1992). Although many studies have been done on the toxin production of *Campylobacter* the current knowledge on the subject is still very limited. *Campylobacter* spp. have been reported to be weakly hemolytic (Arimi et al., 1990) which could play a role in iron acquisition and pathogenesis. Cytolethal distending toxin (CDT) is a heat labile, protease sensitive and nondializable toxin which causes HeLa, CHO, Hep-2 and Vero cells to slowly distend over a period of 3-4 days and then die by a

programmed cell death (apoptosis) (Johnson and Lior, 1984). The CDT production is nearly universal in most of the campylobacter isolates. Cytolethal distending toxins cause sensitive eukaryotic cells to become blocked in the G_2 phase of cell cycle which appears to be permanent (Whitehouse et al., 1998). The role of CDT in *C. jejuni* pathogenesis has not been fully elucidated. However, the activity of CDT suggests a possible contribution to diarrheal diseases. Studies with Caco-2 cells, derived from human colonic epithelial cells revealed that it may have the effect of blocking the development of the mature epithelial cells needed for absorptive functions (Whitehouse, 1998).

1.2.5.7. Lipopolysaccharides (LPS) and Lipooligosaccharides (LOS)

Lipopolysaccharides may be an important adhesin of *Campylobacter jejuni* for cellular and mucus substrates (McSweegan and Walker, 1986). Newell et al. (1985) also identified LPS as a second adhesin of *C. jejuni*. The endotoxic properties of *Campylobacter* LPSs are comparable with those of other enterobacterial LPSs (Moran, 1997). Approximately 40% of the predominant *C. jejuni* serotypes express classical core linked, high molecular weight O antigens (Penner and Aspinall, 1997). The remainder of the serotypes have low molecular weight LOS molecules or other polysaccharide polymers unlinked to the core. In some *Campylobacter jejuni* serotypes, sialylation of the terminal core oligosaccharide sugar creates structures that mimic human gangliosides (e.g., GM1, GD1, GD1a, GD3 and GT1a); antibodies raised against these mimetic molecules are suspected to play a critical role in the development of GBS (Saida et al., 1997).

1.2.5.8. Host factors

Several studies have attempted to demonstrate a role for host membrane carbohydrates as receptors for *Campylobacter jejuni,* but these reports have been inconclusive (Moran, 1995). Oligosaccharide sequences probably play a subordinate role in *C. jejuni* attachment to eukaryotic cells (Stanley, 1989). The addition of simple sugars such as mannose, fucose, glucose, N- acetylglucosamine, maltose and galactose also did not significantly alter the binding of *C. jejuni* to CHO cells. They may interact with lipids in host cell membranes but lipids only partially inhibit *C.jejuni* binding to CHO cells, suggesting that multiple interactions occur between the bacteria and host cells (Szymanski and Armstrong, 1996). Components in intestinal mucus and the extracellular matrix may serve as important receptors for colonization.

1.2.5.9. Microenvironmental factors

Campylobacter jejuni can be isolated from the GI tract of many animals and humans and many environmental sources such as food and water. This demonstrates the ability of these organisms to survive in diverse environments and under stress conditions which is vital for infection and transmission processes. *C. jejuni* is capable of competing well for nutrients such as iron in dynamic environments. There exists a regulatory system in *Campylobacter jejuni* which responds to various microenvironmental factors such as pH, osmolarity, free iron concentration, temperature, and the bacterial growth phase encountered during host infection. The age of the bacterial culture affects adherence of campylobacter organisms to host cells. In older cultures they change their morphology from the typical spiral shape to smaller coccoid forms (Buck et al., 1983). Bacterial growth temperature and surface

hydrophobicity also exerts an effect on adherence (Walan and Kihlstrom, 1988).

1.2.5.9.1. Iron

Iron is the limiting nutrient in the plasma and extracellular fluid of most animals. The proinflammatory cytokines released during a pathogenic challenge mediate a shift in iron from transferrin in the extracellular fluids to ferritin located in intracellular locations (Weinberg, 1995). This change decreases the amount of iron available to pathogens and decreases their virulence.

When colonizing a host, *C. jejuni* must compete with other organisms for available nutrients, including iron. In host tissues the availability of iron is low as most of it is complexed with host proteins such as hemoglobin and transferrin. The low iron concentration constitutes a defense mechanism, but conversely, successful pathogens can use it as a stimulus to express virulence factors like toxins and other factors required for in vivo growth (van Vliet et al., 1998). Iron is the most essential micronutrient for almost all microorganisms including campylobacters. Bacteria in general require 10^{-6} to 10^{-7} M of ferrous iron for survival. These organisms encounter widely fluctuating levels of this essential element and hence should maintain iron homeostasis through the differential expression of iron uptake and storage systems for survival. As a response to nutritional restriction caused by iron limitation, many pathogens have evolved highly efficient systems for sequestering iron from the iron binding proteins, transferrin and lactoferrin found in serum and mucosal surfaces respectively. For this purpose they secrete highly specific chelators for ferric ions called siderophores, which is followed by their reinternalization via a high affinity transport system and subsequent release of iron into the cytoplasm.

Campylobacters have fewer iron uptake systems in comparison with other organisms. Although they do not synthesize siderophores to sequester iron, they are able to bind exogenous siderophores. Systems for enterochelin and, hemin uptake and a transporter for an unidentified siderophore have been characterized in *C. jejuni* (Picket et al., 1992; Park and Richardson, 1995). Although iron is essential for bacteria, its presence within a bacterial cell can be troublesome as it can lead to synthesis of toxic free radicals. Therefore, iron homeostasis has to be balanced carefully by strict regulation of iron uptake and storage. *Campylobacter jejuni* exhibited slower growth rates and altered cellular morphology and synthesized new envelope associated proteins when grown in low iron conditions (Field et al., 1986). Pickett et al. (1992) found that hemin, hemoglobin, hemin- hemopexin and hemoglobin- haptoglobin stimulated growth of *C. jejuni* in low iron medium, whereas transferrin, lactoferrin and ferritin were unable to provide iron to strains tested (Park and Richardson, 1995).

Campylobacters synthesize a series of new proteins in the presence of eukaryotic cells which are important in the invasion. A ferric uptake regulator (Fur) protein has been cloned from *C. jejuni* which has been proposed to control the synthesis of a set of ferrous responsive genes (Wooldridge et al., 1994). Above a certain concentration threshold, ferrous iron can complex with Fur and mediate the formation of a dimer which is able to bind to a palindromic control sequence which overlaps the promoters of Fur regulated genes. For the specific binding of Fur to DNA the presence of one of the following divalent heavy metals, manganese, iron, copper, cobalt, cadmium, and partially zinc is essential (de Lorenzo et al., 1987). During growth under iron replete conditions the Fur proteins bind to the promoter

regions of Fur regulated genes and thereby prevents transcription. Recently, the role of a gene *Nramp1* in the transport of divalent cations such as zinc, iron, and manganese have been demonstrated at the phagosomal membrane of macrophages (Grunheid and Gros, 2000).

1.2.5.9.2. Oxygen

The microaerophilic nature of campylobacters suggests an inherent sensitivity to oxygen and its intermediates. However, campylobacters are able to adapt to aerobic metabolism by producing some changes in colony morphology and outer membrane patterns (Jones et al., 1994). Exposure to oxygen results in the formation of reactive oxygen intermediates which damage nucleic acids, proteins and membranes. The cellular defenses against the damaging effects of oxidative stress may play a major role in the survival of these organisms during exposure to air. Enzymes such as superoxide dismutase, alkyl hydroperoxide reductases, catalase, glutathione synthetase and glutathione reductase seem to provide protection against oxygen stress.

1.2.5.9.3. Temperature

Campylobacter jejuni favor an incubation temperature of 42° C although these organisms are able to grow between a temperature of 30° C and 47° C. The temperature inside the avian GI tract appears to promote the colonization of campylobacters because of this peculiarity. Their ability to regulate gene expression in response to wide variations in temperatures becomes vital for their survival in varying temperature conditions. The expression of virulence factors is a high energy expensive process, so it is highly regulated and limited to periods of invasion and colonization only. The movement from the environment to the host necessitates expression of several virulence factors. Increase in the

temperature acts as one cue for the expression of virulence factors.

Campylobacter lack many of the classical environmental regulatory proteins that are encountered in other bacteria. This is quite intriguing because they are able to survive in diverse environmental conditions such as the GI tract, food and aquatic environments, suggesting the possibility of these organisms possessing unique systems for environmental regulation not encountered in other organisms. In addition, several two component regulatory systems have been found in *C. jejuni* (Ketley, 1997). These systems consist of a membrane histidine kinase sensor which is activated under appropriate environmental conditions and in turn phosphorylates a diffusible response regulator protein that is needed to initiate the transcription of specific genes. Expression of some genes such as *fla B* is controlled by unique genetic promoter sequences that are environmentally modulated by temperature, pH or divalent cation levels (Alm et al., 1993). The expression of bacterial factors affecting adhesion and invasion are controlled by a series of different regulatory mechanisms that are modulated by environmental factors.

1.3. Interaction between dietary factors and colonization of microflora

1.3.1. Issues related to the use of antimicrobial agents in the diet

Today's consumers demand safe, wholesome and at the same time nutritious food products which do not adversely affect their health. Not only the health and welfare of animals but also the economics of the food animal production industry is very much dependent on drugs, especially antimicrobial agents (NRC, 1999). While beneficial for animals and to the industry, drug use is not without adverse human health effects. Both experts in the field and the general public are aware of the consequences of antibiotic,

pesticide, and toxic waste residues in foods, their environmental impacts and emergence of microorganisms resistant to antimicrobial agents and are concerned about their presence in foods (NRC, 1999).

Pathogenic organisms such as *Salmonella* DT-104, *E. coli* O157:H7 and *Campylobacter jejuni* are becoming increasingly important to human health due to their pathogenicity and added resistance to antimicrobial agents used in human medicine. With each additional acquisition of resistance to yet another class of antimicrobial agents, the treatment of that infection becomes more difficult and hence the potential risk to human health becomes greater. The main source of infection is food of animal origin. Both *Campylobacter* and *Salmonella* spp. colonize the GI tract of poultry and contaminate food. The USFDA recently announced its plans to ban the use of two antibiotics (enrofloxacin and sarafloxacin) in the fluoroquinolone family that are used in poultry production because the use of these drugs increases the likelihood of human infection with fluoroquinolone resistant strains of *C. jejuni,* the nation's most common cause of bacterial foodborne illness (Cimons, 2001). The USFDA is also considering whether they must assess the likelihood that the use of a certain drug in food animals will transfer resistance and create a public health problem before it reviews a new animal drug for approval. Therefore, on a public health perspective it becomes necessary to reduce the unwarranted use of antimicrobial agents in food animals. At the same time, there is a need for preserving the health and productivity of animals. For this, natural alternatives need to be developed as feed additives for animal agriculture.

Appropriate strategies should be aimed at enhancing growth and productivity of food animals by providing dietary nutrients in optimal amounts at appropriate times of need to

meet the demands and at the same time avoid strains on the system for a particular demand. As the trend among consumers to pay more for safer food products is increasing, the organic approach of producing poultry meat is worth trying (NRC, 1989).

Bacteria in the digestive tract play an important role in the metabolism in the gut lumen (Savage, 1986), but the significance of GI microflora in the nutrition of chicken and the role played by dietary factors in the colonization of microflora have not been fully understood.

1.3.2. Gut microflora in birds

The GI flora of non-ruminants are usually more commonly found in the lower GI tract. Their population varies widely in composition and activity and distribution in the various regions of the GI tract. These variations are a result of different conditions that are affected by factors such as diet composition, dry matter content, and acid and alkali secretion in the digestive tract (Savage, 1983). In birds, the ceca provides a stable environment for microbes and so harbors the largest and most complex of GI microbial ecosystems.

The intestinal tracts of freshly hatched chickens are normally sterile, but appreciable numbers of bacteria are detected within a few hours. Immunoglobulins A and Y play important roles in the selective colonization of GI tract of birds following hatching. Immunoglobulin A is particularly important in determining the microflora that will be permitted to colonize the GI tract (Klasing, 1998). It takes about two weeks for the bacterial population to get established in the small intestine, but more time is needed for the cecal ecosystem to develop and about 6-7 weeks are needed for its full development (Smith, 1965). This may suggest that broiler chickens marketed by about five weeks of age are very unlikely

to attain a stable cecal microbial ecosystem.

There occurs a constant selection of microflora that grow and colonize the intestinal tract to produce a flora specific to a particular host species. Several inhibitory compounds of a chemical nature, such as volatile fatty acids, bile acids, hydrogen sulfide and immunoglobulins act against the colonization of bacteria in the GI tract (Fuller, 1984). Different bacterial species are found in different parts of the gut, as the requirements of growth for these species vary greatly. Gram positive organisms such as *Lactobacillus* and *Streptococcus* spp. predominate the crop and stomach. In the small intestine, mainly aerobes, gram positive *Enterococcus*, *Lactobacillus* and *Bifidobacterium*, facultative anaerobes, gram negative coliform bacteria and strictly anaerobes, gram positive clostridia and gram negative *Bacteroides* are found. *Bacteroides* and *Fusobacteria* are found mainly in the ceca (Mead, 1989). The intestinal ecosystem is considered to be more stable when the number of bacterial species in the GI tract is high (Mead, 1989). As young birds have fewer species of bacteria than adults, their ecosystem is less stable and so its balance could be easily disturbed. Environmental differences between young and adult may also influence the colonization of bacteria.

1.3.3. Intestinal microflora on health status

The mammalian intestine appears to tolerate most intestinal microbes which are antigenically foreign. This tolerance could be due to some advantages offered by microflora to the host. There exists a constant dynamic equilibrium between the indigenous bacteria entering the body and the systemic defense mechanisms eliminating these invaders (Freter et al., 1983). The primary functions of microflora are nutritive, metabolic, immunologic and

protective in nature. A healthy GI flora forms a barrier against invading organisms. They can enhance the host's defense mechanisms against pathogens and can improve intestinal immunity by adhering to intestinal mucosa and stimulating local immune responses (Salminen et al., 1995).

Normal indigenous GI microflora protect animals and humans from the invasion of pathogenic organisms to a certain extent. They play a major role in stimulating immunological host defense mechanisms. The constant penetration of indigenous flora gives rise to the normal antibodies which are probably the most important markers for nonself recognition in phagocytosis. It was observed that addition of *Lactobacillus* spp. to the diet of pigs stimulated the production of antibodies and phagocytic activity against pathogenic bacteria in the intestine. This effect is due to increased secretion of IgA which provides a primary line of defense against pathogens (Fuller, 1989).

Dietary supplementation of certain specific bacteria may therefore help the animals to combat infections. The composition of the indigenous microflora thus controls to a considerable extent the spectrum of microbes which the body can eliminate efficiently via phagocytosis (Freter, et al., 1983). A part of the autochthonus GI mucosal microflora, the segmented filamentous bacteria (SFB) have been found to provide the single most potent microbial stimulus for the murine gut mucosal immune system (Talham et al., 1999). According to Lanning et al. (2000) certain currently undefined intestinal microflora are required for diversification of the antibody repertoire. Germ free or antibiotic treated experimental animals have been shown to be more susceptible to infections than conventional animals (Henteges, 1979).

The GI microflora in humans provide the host with nutrients such as short chain fatty acids, vitamin K, some B vitamins and amino acids (Savage, 1986). They detoxify toxic materials such as heterocyclic aromatic amines to some extent (Roberfroid et al., 1995). They may play either a beneficial or detrimental role in the promotion and progression of neoplastic cells in the carcinogenic process. Primary functions may be in the absorption and metabolism of mutagenic and carcinogenic chemicals. Secondary roles may be via butyric acid, bile acids or diacylglycerols (Roberfroid, et al., 1995). Changes in normal human GI flora result in the development of intestinal disorders. Pathogenic bacteria alter the intestinal microecology and intestinal colonization resistance (Salminen et al., 1995).

A well balanced composition of microflora provides protection against invading pathogens and also helps the birds to obtain an optimal nutrient composition (Salminen et al., 1995). Fermentation of nondigestible dietary substrates as well as of endogenous mucus is a major metabolic function of microflora. The main products of such anaerobic oxidations are gases, hydrogen, carbon dioxide, short chain fatty acids (SCFA), lactic acid, ethanol, branched chain fatty acids (BCFA), ammonia, amines, phenols, and indoles. Gases are ubiquitous products of anaerobic microbial metabolism. The SCFA are the metabolic endpoints of carbohydrate fermentation, whereas the BCFA result mainly from the oxidative breakdown of amino acids. In humans the endpoints of fermentation serve the nutritive function of microflora. Short chain fatty acids and lactic acid are absorbed and serve as energy substrates. They may serve as modulators of glucose and lipid metabolism. The SCFA may stimulate the absorption of calcium, magnesium, and iron from the colon in humans (Roberfroid, et al., 1995).

The inhibition of growth or reduction in number of one bacterial species by one or more other bacterial species is called bacterial antagonism. The antagonism involves direct and indirect mechanisms. Indirect antagonism involves modification of bile salts, induction of immunologic processes and stimulation of peristalsis. Direct mechanisms are depletion of or competition for essential substrates, competition of bacterial receptor sites, creation of a restrictive physiologic environment and elaboration of an antibiotic like substance (Rolfe, 1991). The mechanism of bacterial antagonism is taken advantage of in the competitive exclusion (CE) or colonization resistance strategies (Nurmi and Rantala, 1973).

1.3.4. Environmental factors that modify microbial growth

The factors that affect the environment are interrelated and their combined interactions would determine the organism that may be favored. The factors include pH, nutrient content, physical and chemical properties of diet, temperature, moisture availability, oxidation reduction (OR) potential and presence of certain inhibitory substances (Rolfe, 1984; Freter et al.; 1983; Henteges, 1970). Each microbial organism has its own characteristic ability to utilize certain substances as sources of energy, carbon or nitrogen and the chemical composition of food determines how it will support the growth of microorganisms.

1.3.4.1. Temperature

Temperature, the most important environmental factor affecting the growth and viability of microorganisms affects the duration of lag phase, the rate of growth, the final cell numbers, the nutritional requirements and the enzymatic and chemical composition of cells. The influence of temperature on the activity of microorganisms is greatest in moist feeds at

water activity (explained below) above 0.85 (Olson and Nottingham, 1980).

Heat may change the physical and chemical structure of feed. It may soften the tissues, release or bind water, destroy or form colloidal suspensions, emulsions or change the access of feed to moisture or oxygen. Proteins may be denatured and protein may become more available to some organisms. Starch and proteins may be gelated, releasing moisture and becoming more easily decomposed (Christian, 1980).

1.3.4.2. pH

Each microbial organism prefers an optimal pH, but microbial cells could be affected by the pH of diet as they do not have any mechanism for adjusting their internal pH (Corlett and Brown, 1980). Diets with very low pH do not support bacterial growth well. pH is one of the decisive factors for the development of microbial communities, and even slight variations can greatly influence the development of microbial flora (Sinell, 1980). Microbial growth tends to change pH. Acids are produced mainly by dissimilation of carbohydrates, whereas alkaline conditions are the result of production of ammonia or amines with the breakdown of proteins.

Bacteria which reduce pH include lactic acid bacteria, *Acetobacter*, *Gluconobacter*, and *Pediococcus* species. Examples of organisms that raise pH include *Pseudomonas*, *Alcaligenes*, *Acinetobacter* and *Moraxella* species. Most bacteria are favored by a pH near neutrality. Tolerance of acid or alkaline conditions is usually but not always related to acidogenesis or alkaligenesis. Acid forming organisms are favored by moderate acidity whereas proteolytic bacteria are favored by an alkaline pH. *Acetobacter* and *Lactobacillus* spp. tolerate pH values that are inhibitive to other food bacteria. Buffers present in the feed

may resist fluctuations in pH and prolong either an acid or alkaline fermentation. Proteins are good buffers which permit considerable growth and acid production by lactic acid bacteria.

1.3.4.3. Water activity (a_w)

The water in food, its location and availability greatly influence microbial growth. All microorganisms have a requirement of water for their growth and the water should be in the available form. The water requirement is expressed in terms of available water or water activity (the vapor pressure of the solution divided by vapor pressure of the solvent) (Christian, 1980). Most bacteria require an a_w of above 0.90. If the a_w is reduced below the optimal level, there occurs a lengthening of the lag phase of growth and a decrease in the rate of growth (Christian, 1980). It is likely that microorganisms can change the level of available moisture by changing the substrate to free water. For example, *Bacillus subtilis* can release water by decomposing starch and make conditions favorable for its own growth.

1.3.4.4. Oxidation reduction (OR) potential (*Eh*)

The oxidation reduction potential or oxidizing and reducing power of the feed could influence the type of organisms that grow. The OR potential of a feed is determined by its inherent OR potential, the poising capacity (the resistance to change in OR potential of the feed), the oxygen tension of the atmosphere of the feed, and the extent of access the atmosphere has to the feed. In a weakly poised feed a small microbial population may possibly cause a large change in redox potential whereas in a heavily poised feed a large population may scarcely affect the OR potential (Brown and Emberger, 1980). A high OR potential favors aerobes and facultative organisms while low OR potential, anaerobes or

facultative organisms.

Growth of an organism may alter the OR potential of a feed so that the growth of other organisms may be inhibited. For example, the growth of facultative anaerobes and clostridia in the chick's GI tract has been reported to lower the OR potential and promote growth of anaerobic organisms that produce VFA and other substances that inhibit the growth of enteropathogens (Barnes et al., 1980). Low OR potentials have been reported to increase the antibacterial activity of VFA (Pongpech and Henteges, 1989). The degree of VFA undissociation increases as the pH of the acid environment decreases (Meynell, 1963). Acids are then able to enter the bacterial cell and inhibit bacterial metabolism. The OR potential (*Eh*) is expressed and measured in terms of millivolts (mV). Aerobic organisms such as *Bacillus*, *Micrococcus*, *Pseudomonas* and *Acinetobacter* require positive *Eh* values while anaerobes such as *Clostridium* and *Bacteroides* require negative *Eh* values. Most fresh plant and animal tissue have low and well poised OR potential in their interiors. This is because of the presence of reducing substances such as ascorbic acid and reducing sugars in plants and sulfhydril groups in animal tissues.

1.3.4.5. Inhibitory substances

Certain microorganisms growing in feeds may produce substances such as hydrogen ions, carbon dioxide organic acids, alcohols, peroxides and antibiotics which are inhibitory to other organisms. Propionibacteria produce propionic acid which is inhibitory to molds. *Streptococcus lactis* produce a substance called nisin which is inhibitory to lactate fermenting gas forming clostridia. Some microorganisms are capable of destroying inhibitory compounds in feeds. Certain molds and bacteria can destroy phenol compounds, benzoic

acid, and nisin. Heating lipids may enhance autooxidation and make them inhibitory. Peroxide formed during oxidative breakdown of lipids and also as a metabolite of carbohydrate dehydration are inhibitory to most microorganisms (Sinell, 1980).

1.3.5. Dietary factors and substrates that influence microbial colonization

Nutrient diversity and digestibility are the dietary factors that could influence the ecosystem of the GI tract, especially in the ceca and the large intestine (Lee, 1985). The microflora may also compete for nutrients. This competition may be in favor of the host if the diet is highly digestible. However, when the diet is poorly digestible, more substrates move to the lower part of GI tract thus favoring the growth of microflora. As the digestive capacity of young birds would not be fully developed, the digestion of nutrients would not be efficient, thereby, leaving more substrates for microbial fermentation following digestion (Nitsan et al., 1991; Nir et al., 1993).

1.3.5.1.Carbohydrates

The carbohydrate components of diets include sugars, disaccharides, starch and NSPs. The carbohydrates, especially sugars, are most commonly used as an energy source. But esters, alcohols, peptides, amino acids, and organic acids and their salts also may serve as carbon sources. Starch and complex carbohydrates such as cellulose are utilized by very few microorganisms. Also, microorganisms differ in their capacities to utilize even simple sugars. For instance, many organisms cannot utilize lactose. Wilkins (1981) observed that the loss of fusiform organisms from both the lumen and the mucous layer of the large intestine of rodents was accompanied by a large increase in the population of facultative enteric bacilli, and cellulose was the only component that resulted in the maintenance of the

fusiform flora.

Mannose, a monosaccharide, reduces colonization of *Salmonella typhimurium* and *E. coli* by blocking the mannose specific receptors on the surface of these pathogens (Oyofo et al., 1989). The blocking action, however, of D- Mannose on *E. coli* adherence to epithelial cells is reversible (Ofek et al., 1977). Addition of lactose to the diet increases the lactic acid and VFA content in the intestine and ceca which causes a decrease in cecal pH (Corrier et al., 1990; Hinton et al., 1990). Lactose also reduces colonization of pathogens by virtue of specific affinity of bacteria for this specific carbohydrate (Hinton et al., 1991; Nisbet et al., 1993). Lactose stimulates the growth of certain specific bacteria, in particular that of *Lactobacillus* spp. which prefers it as a carbohydrate source (Morishita et al., 1982).

1.3.5.2. Water soluble viscous NSPs

Dietary fiber is composed of nonstarch polysaccharides and lignin, which cannot be digested autoenzymatically. Structurally they are composed of fibrillar polysaccharides (cellulose), matrix polysaccharides (hemicellulose and pectin) and encrusting substances (lignin). Functionally, fiber is subdivided into soluble (pectins, gums and β- glucans) and insoluble (lignin and cellulose). Beta glucans of barley and arabinoxylans and pentosans of wheat and rye are the major water soluble NSP fractions. Studies by Hofshagen and Kaldhusdal (1992) and Riddle and Kong (1992) indicated that presence of water soluble NSPs in the diet could increase microbial activity in the GI tract especially that of clostridia and enterobacteria. Microbial growth could be promoted by increased levels of undigested nutrients in the lower parts of the small intestine. An increased flow of undigested nutrients to the end of the small intestine promotes microbial activity (Choct et al.,1992).

1.3.5.3. Fats

Only very few microorganisms can utilize fats and they do so only if a more readily available energy source is absent. This is because glycolysis and proteolysis usually proceed much more rapidly than lipolysis as carbohydrate and protein substrates are often soluble and so readily accessible to microbial enzymes. But the fats are relatively insoluble so that the amounts directly accessible to microbial enzymes are very limited (Sinell, 1980). First the fats are hydrolyzed to glycerol and fatty acids by lipase. Aerobic microorganisms are commonly involved in the decomposition of fats and lipolytic organisms are usually proteolytic also. The bile acids which are essential for digestion and absorption of dietary fats in the intestinal tract, may play an important role in regulating the composition of the normal intestinal microflora (Savage, 1977). The processes involved in the degradation of bile acids, dehydration, deconjugation and dehydrogenation can change the efficiency of solubilizing fats (Hofmann and Mysels, 1992). Several intestinal organisms such as *Enterococcus*, *Bifidobacterium*, *Clostridium*, *Bacteroides*, *Lactobacillus* are capable of catalyzing the deconjugation of bile acids in vitro (Grill et al., 1995), but their behavior in vivo is not known.

Polyunsaturated fatty acids (PUFA) can alter the microbial colonization by influencing the functioning of the immune system considerably. Immune cells use PUFA to produce tissue hormones known as prostaglandins. Diets rich in n–3 PUFA tend to inhibit the immune response whereas those rich in n–6 PUFA tend to promote immune responses that lead to inflammation. n-6 series are found in vegetable oils while n-3 series are found in fish oils and vegetable oils such as canola, soy and linseed (Meijer, 2001).

1.3.6. Feed additives

Feed additives are substances added to feeds to improve their flavor, color, texture or keeping quality and performance of livestock, including poultry. Substances commonly used as feed additives in poultry are antibiotics, enzymes, prebiotics and probiotics.

1.3.6.1. Antibiotics

Antibiotics in subtherapeutic doses have been used as feed additives in order to modify weight gain, feed utilization and protection against infections especially in young animals. Antibiotics used as feed additives help to enhance the utilization of dietary energy (March et al., 1978). They reduce the number of gram positive bacteria (MacKinnon, 1985). Antibiotic growth promoters remove the bulk of the flora directly by interrupting their ability to replicate or by killing them directly.

1.3.6. 2. Enzymes

The commonly used exogenous enzymes in poultry feed are xylanases, β-glucanases and phytases. The benefits of enzyme use are brought about through an increase in digestibility of the fiber component in the diet. As a result of such improvements in diet digestibility, there may be significant change in the substrate quality and quantity available to the intestinal microflora (Bedford, 2001). Enzymes function by increasing the rate of digestion such that there is less substrate available to support the microflora. Enzymes are more effective when the quality of the cereal used is low. Poor quality cereals contain greater quantities of the anti-nutritional factors that the relevant enzymes target, and as a result the benefit upon enzyme utilization is greater in such diets (Bedford, 2001). This benefit is related to microbial population which result from enzyme supplementation. Sharma et al.

(1997) observed that a diet supplemented with xylanase, led to changes in intestinal viscosity and mucin composition. Xylanase supplemented diet lowered the viscosity but increased the amount of neutral, carboxylated and sulfated mucins in the jejunum. However, according to Shapiro and Nir (1995) enzyme supplementation had no effect on feed intake, growth or feed utilization or on digestibility of fat, starch, protein or energy.

1.3.6. 3. Prebiotics, probiotics and synbiotics

Prebiotics are oligosaccharides that are not hydrolyzed in the small intestine but at the same time modify the composition of microflora in the large intestine. The objective behind prebiotics is to promote the growth of specific beneficial bacteria such as *Bifidobacterium* or *Lactobacillus* or to reconstitute the composition of microbial ecosystem. It appears that *Bifidobacterium* has the greatest ability to ferment oligosaccharides. It would provide support for the existence of an opportunist relative to autochthonus strains and therefore enhance concepts of microbial ecology of the intestinal tract (Tannock, 1999).

Probiotics (for life), according to Fuller (1989) consist of live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance. The major components of probiotics commonly used in farm animals are *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Bacillus*, yeasts and filamentous fungi such as *Saccharomyces*, *Aspergillus* and *Torulopsis* (Berg, 1998). The USFDA require that the manufacturers of these products use the term direct fed microbial (DFM) instead of probiotic. Synbiotics are probiotics and prebiotics used in combination. Examples are *Bifidobacterium* with fructooligosaccharide and *Lactobacillus* with lactitol. These combinations could improve the survival of the probiotic organism because its specific

substrate is readily available for fermentation and results in advantages to the host that the live microorganism and prebiotic offer (Collins and Gibson, 1999). However, the results with respect to the effects of pre and probiotics on broiler performance and nutrient utilization are quite variable and unpredictable.

1.4. Designing diets for poultry

Broiler chickens have been selected for rapid weight gain and efficient feed utilization. They are usually allowed to eat *ad libitum* to ensure rapid development. Many nutrients are interdependent, and it is difficult to express requirements for one without consideration of the quantity of the other (NRC, 1994). The main focus on designing a ration for poultry should depend on the quality of feed because feed passes through the digestive tract quickly. Therefore, the feed should contain not only all required nutrients but they also should be in readily available form. The basic principle behind diet formulation is that birds eat primarily to satisfy their energy requirements. Energy is supplied by the grain in the diet. If adequate protein is available some amino acids will be synthesized in the body. The amount of essential amino acids and their availability is a measure of the quality of the protein supplement. In the case of essential amino acids, greater dietary concentrations may be required to optimize efficiency of feed utilization than would be needed to maximize weight gain. The dietary requirements for lysine to maximize yields of breast meat of broilers are greater than that needed to maximize weight gain (Acar et al., 1991).

1.5. Proteins and amino acids

Chickens do not require a specific level of crude protein per se; rather, they have a requirement for specific amino acids plus sufficient protein to supply either the nonessential

amino acids themselves or amino nitrogen for their synthesis (NRC, 1994). Relatively high concentrations of dietary amino acids are needed to support the rapid growth of broiler chickens. Body weights of broiler chickens will increase approximately 50 times after hatching and a major increase in weight is due to tissue of substantial protein content. Therefore, adequate amino acid nutrition is essential for successful feeding programs for broiler chickens. There are 9 essential amino acids which cannot be synthesized in the body. Histidine, glycine and proline can be synthesized, but the rate of synthesis is not sufficient to meet the metabolic demands of growing birds and hence should be supplied in the diet along with the essential amino acids. However, the greatest requirements are for methionine and cystine. The requirements for methionine can be satisfied only by methionine whereas that for cystine can also be met with methionine. The other important amino acids required are arginine, lysine, tryptophan, threonine, isoleucine, leucine, valine, phenylalanine plus tyrosine, glycine plus serine, histidine and proline. The NRC (1994) recommendations for meeting the requirements for protein and various amino acids are given in appendix A.

A protein that has a balance of essential amino acids that exactly matches a bird's requirements along with sufficient nonessential amino acids is referred to as an ideal protein (Cole and Van Lunen, 1994). Real world foods deviate from an ideal balance of amino acids, resulting in excesses of certain essential and nonessential amino acids, which are lost to catabolism. The greater the deviation in amino acid balance of dietary protein from that of an ideal protein, the higher the level of protein needed to meet the bird's amino acid requirement (Klasing, 1998). In application, the amino acid content of foods should be corrected for its digestibility because the published amino acid requirement values are valid

only for highly digestible proteins. Fat extracted feedstuffs, such as soybean meal have highly available protein content.

The most available amino acids from animal proteins are isoleucine, lysine and threonine (Yamasaki and Kamata, 1988). Animal protein sources are economical feed ingredient for poultry as they contain high quality proteins, critical amino acids, available phosphorus and minerals. Poultry by-products meal can provide all essential energy and other nutrients. Feather meal is quite deficient in methionine, lysine, tryptophan and histidine. Fish meal is an excellent protein supplement high in lysine and methionine (Patrick and Schaible, 1980a).

On the other hand proteins from most plant sources are not adequate in essential amino acids. The most limiting amino acids are lysine, methionine and cystine. The most available amino acids are histidine, arginine, leucine and phenyl alanine (Yamasaki and Kamata, 1988). Soybean meal is a good source of proteins, rich in lysine and tryptophan but deficient in methionine. Corn gluten is low in lysine and tryptophan. Protein complementation by adding soybean to corn corrects the deficiency of corn. Corn-soybean meal diet are still deficient in methionine and cystine (Patrick and Schaible, 1980b). Canola meal is a good source of proteins. The content of amino acids lysine, methionine and cystine is better than that of soybean. So when a vegetable based feed is formulated the ingredients should be varied, or synthetic amino acids should be added to correct amino acid deficiencies. Since the most critical amino acids are methionine and cystine they were added to the vegetable protein based and the animal protein based diets respectively, to balance their requirements.

1.6. Rationale for the study

The main objective of this study was to evaluate how feed formulations containing ingredients of vegetable origin and animal origin influence the colonization of *C. jejuni* in the intestinal tract of broiler chickens. Certain plant components such as complex carbohydrates when included in the diet were found to promote fermentation processes in the ceca of birds (Hofshagen and Kaldhusdal, 1992; Riddle and Kong, 1992). The products generated from the fermentation processes modulate the microenvironment of the ceca. Since the pathogen, *C. jejuni* primarily colonizes the ceca of birds, it was presumed that any changes taking place in the microenvironment of the ceca would have an effect on the colonization of this organism.

Another important factor that is likely to influence the colonization of *C. jejuni* is the available iron content of feeds. In plant based diets the iron is found mostly in bound form, which is not freely available (Freeland-Graves, 1988). Whereas, the animal origin feeds are rich sources of heme iron which is readily available (Walter, 1997). Low iron environment supports growth of lactobacilli which use cobalt or manganese instead of iron. So the difference in the available iron content between vegetable based diets and animal based diets is likely to influence the colonization of *C. jejuni*.

In order to test this, two basic diet formulations were designed; one containing essentially animal protein sources and the other with vegetable origin protein sources. The animal protein components used in the diets consisted of meat meal, poultry by-products meal, fish meal and feather meal. The sources of vegetable protein components used in the diets were soybean meal, canola meal and corn gluten meal (vide Table 2.1).

1.7. Research hypothesis and objectives

The general hypothesis of this project was made based on the current knowledge on the unique features of colonization of *Campylobacter jejuni* in chickens and nutrition and digestion in chickens. The organism is ecologically adapted to the avian intestinal tract and has selected the ceca as a special niche for colonization. Although the digestion in birds is simple, a little fermentation takes place in the lower intestines especially the ceca where the microenvironment is ideal for the process when certain substrates are present in the diet. It was therefore hypothesized that, *C. jejuni* being a nonfermenter, diet formulations that differ in type or nature of ingredients could alter the microenvironment within the ceca, which would influence the colonization of this organism.

The long term objective was to reduce the risk of transmission of the food borne organism *C. jejuni* from the intestinal tract of broiler chickens to humans. The goal of this research was to examine how differences in diet due to protein sources (animal origin or plant origin) influenced the intestinal colonization of *C. jejuni* in broiler chickens.

The specific objectives were:

1. To gain information on the intestinal colonization of *C. jejuni* in broiler chickens and to determine how proteins of animal origin and plant origin influence the intestinal colonization of these organisms. Do changes in feed composition influence the colonization of these organisms in broiler chickens?
2. To characterize the *C. jejuni* isolates recovered from birds in the experimental group to determine whether the isolates recovered and strain used for challenge were identical. This was performed using standard methods, biotyping and serotyping

methods.

3. To study the antibiotic sensitivity patterns of the isolates and to determine the minimum inhibitory concentrations using E- test^{R} (AB Biodisk, Sweden) and the effects of different diet compositions on the sensitivity patterns.

4. To genotype the isolates using *fla A* typing with the Restriction Fragment Polymorphism (RFLP) of the Polymerase Chain Reaction (PCR) products of *fla A* gene.

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2. THE EFFECTS OF DIET FORMULATIONS CONTAINING PROTEINS FROM DIFFERENT SOURCES ON THE COLONIZATION OF *CAMPYLOBACTER JEJUNI* IN THE GASTROINTESTINAL TRACT OF BROILER CHICKENS

2.1. Background

Poultry, both wild and domesticated, are asymptomatic carriers of *Campylobacter* spp. in their intestinal tract (Skirrow, 1982). The organisms are transmitted onto poultry meat during mechanized processing because of heavy intestinal carriage and possible rupture of intestines during evisceration. Studies by Ooestrom et al. (1984) and Deming et al. (1987) suggested that poultry are responsible for more than 50% of human campylobacteriosis. The association between birds and *Campylobacter* is harmful not only because of public health concerns but also because of its potential for economic damage to the industry. The general consensus is that the best way to reduce public health hazards due to *Campylobacter* is to reduce the colonization of these organisms in the gastrointestinal tract of birds while they are growing. A reduction in intestinal colonization will reduce carcass contamination and improve the microbiological safety of poultry products for the consumer.

Current research is focused on three approaches; (i) Production intervention at farm level using strategies such as competitive exclusion (CE); (ii) Utilization of genetic variation of birds (differences in colonization susceptibility between different commercial lines of broiler chickens); (iii) Immunological intervention (development of a vaccine). Our approach was directed at a farm level production intervention, thereby reducing the intestinal carriage of *Campylobacter jejuni* in broiler chickens.

The hypothesis was that differences in diet formulations due to variations in the type or

nature of ingredients (using proteins from different sources) would influence the colonization of *C. jejuni* in the intestinal tract of broiler chickens.

2.1.1. Nutritional modulation of immune response and infection

The response of an animal to infections depends on its immune status, which in turn depends in part on its nutritional status (Tengerdy et al., 1990). Nutrition affects the development of the immune system as well as the immune response to disease. There is a bidirectional influence of nutrition and infection in which malnutrition may predispose the animal to infection or increase the severity of infection and infection itself may result in nutritional abnormalities (Keusch, 1998). The nutritional status of the developing embryo has profound effects on the development of its immune system. Nutritional status during embryonic development may affect immunocompetence for the life of the animal. The immune response to a pathogen can affect the nutrition of animals by altering digestion, absorption, metabolism and excretion of nutrients thus affecting substrate availability and dietary requirements (Koutsos and Klasing, 2001).

Components of the immune response may increase nutrient use, the anabolic components of the immune response such as clonal proliferation of lymphocytes, recruitment of new myeloid cells and antibody synthesis as well as acute protein synthesis by the liver (Koutsos and Klasing, 2001). Nutrients provide the substrates necessary for proliferation of immune cells and effector molecules. In the absence of adequate nutrients the immune response to diseases may be insufficient. A nutrient deficiency or excess may enhance the virulence of pathogens. With sufficient knowledge of the interactions between nutrition and immune response, the producer can optimize productivity and welfare while

minimizing disease concerns and the cost of over or underfeeding animals (Koutsos and Klasing, 2001). Improvement of animal health by nutritional means will reduce the use of antimicrobial agents in animal production and at the same time provide humans with safe food.

Although the literature contains many reports on dietary components altering immune function, the conclusions of these studies may not be always reliable due to the lack of repeatability in many cases (NRC, 1999). Since most of these studies were conducted in vitro, they do not reflect true in vivo interactions. In order to obtain optimal disease resistance, animal diets must be well balanced and formulated for the appropriate stage of growth and production. The development and expression of the regional immune system in the GI tract is relatively independent of systemic immunity.

Diet can influence resistance to infection because specific nutrients are important in endocrine regulation, immune and somatic cell function, antibody production and tissue integrity (Tengredy et al., 1981). The concentrations of some micronutrients such as iron and zinc are very low in body fluids, making them the limiting substrates for the proliferation of invading pathogens. The iron binding protein (transferrin) mediates the transfer of iron out of blood plasma and into the liver where it is less available to pathogens (Hallquist and Klasing, 1994). However, there is another school of thought, that iron limitation of the body need not be to deprive pathogens of their substrate but a physiologic reality because iron is such a reactive metal with capacity to severely damage membranes and DNA through catalysis of oxidative and peroxidative reactions (Keusch, 1998).

2.1.2. The relationship of proteins with immune response and infection

Protein and energy are important for maintenance of proper health status. Most defense mechanisms are impaired in protein-energy malnutrition, even if the deficiency is only moderate. There is a protein minimum for optimal immune function (Parker and Goodrum, 1990). Protein malnutrition is a major contributor to the morbidity and mortality from infectious diseases, largely as a result of impaired immune responses (Chandra, 1991). During infections the general pattern observed is decreased feed intake with simultaneous increased demand for proteins (Beisel, 1985). During infection extra protein is needed for gluconeogenesis and synthesis of new proteins for the host's defense mechanism (Adams et al., 1996). The acute phase response is a more significant consumer of nutrients than the immune system itself and acute phase response is attenuated in protein deficient humans as well as rodents (Woodward, 1998). Protein deficiency results in decreased acute phase protein synthesis and decreased nitric oxide production in rats (Wu et al., 1999).

The synthesis of acute phase proteins is under the positive control of proinflammatory cytokines interleukin-1 (IL-1), IL-6 and tumor necrosis factor -α (TNF-α) (Kushner and Mackiewiez,1993). These proinflammatory cytokines are thought to be responsible for the metabolic changes elicited by infection (Johnson, 1997). Proinflammatory cytokines stimulate neurons that inervate immune tissues such as lymph nodes, spleen, bone marrow or liver, permitting communication with areas of the brain that regulate important processes such as body temperature, appetite and behavior. Stimulation of local neuronal network modifies vascular tone in specific tissues and in some cases decreases GI motility which affects feed intake. Bacterial pathogens tend to stimulate production of

proinflammatory cytokines whereas viral pathogens stimulate interferons (Koutsos and Klasing, 2001). Production of cytokines and the metabolic changes they induce are important in the adaptive response to infection, and it is also clear that overproduction of these molecules could have potentially deleterious effects (Webel et al., 1998). Webel et al. (1998) have found that administration of vitamin E could attenuate the elevation in plasma IL-6 and cortisol caused by a challenge dose of LPS in pigs.

According to Klasing et al. (1987) administration of a variety of immunogens, such as *Escherichia coli, Salmonella typhimurium* endotoxins, heat killed *Staphylococcus aureus* and the inflammatory polydextran, sephadex, resulted in lower rates of growth and lower efficiency of feed utilization compared with saline injected control chicks. Decreased feed intake accounted for about 70% of the decreased growth with the remainder due to metabolic inefficiencies inducted by immune response. These changes are thought to be orchestrated by IL-1 and possibly by other hormones and cytokines (Klasing et al.,1987). These types of investigations help to understand the risk of bacterial colonization on mucosal surfaces. Lower rates of productivity during disease result in decreased nutrient requirements. In growing chickens (Klasing and Barnes, 1988) and pigs (Webel et al., 1998) the overall requirements for lysine were found to decrease during a simulated infectious challenge. Presumably, this is because lower rates of growth and muscle accretion decrease amino acid requirements much more than are necessary to compensate for the anabolic processes of the immune response (Koutsos and Klasing, 2001). Proteins are needed for increased skeletal muscle catabolism and hepatic protein synthesis during inflammation, infection and trauma.

There are experiments which vary the amounts of single amino acids in diets with results which suggest there are specific single amino acids which are important for optimal immune function. Mice fed diets limited in amino acids tryptophan, leucine, lysine, histidine, methionine or threonine showed a difference in susceptibility to pathogens which require T cell dependent processing by the cell mediated immune system. A deficiency of phenylalanine results in a decrease in antibody production and the number of antibody producing cells (Bounos, 1978). The amino acid cysteine is needed for increased protein production and glutathione production (Malmezat et al., 1997). T cells are especially affected, resulting in their low circulating numbers (Meijer, 2001).

Deficiency of protein decreases glutathione production in pigs (Jahoor et al., 1995). Glutathione (L-gamma glutamyl L-cysteinyl glycine) is an important tripeptide which in its reduced form has protective functions. It protects other intracellular molecules from oxidation, crucial for detoxification of endogenous toxins, elimination of exogenous toxins and xenobiotics and its antioxidant property makes it an important factor in the optimal immune function of macrophages and lymphocytes (Bounous and Gold, 1991). Arginine deficiency results in decreased nitric oxide synthesis in rats (Wu et al., 1999). Glutamine is essential for gastrointestinal epithelium and associated lymph nodes (Yoo et al., 1997). On the other hand an excess of proteins may also be undesirable. An excess of amino acid phenylalanine can result in a decrease in the number of antibody producing cells (Bounos, 1978).

Most of the research efforts in nutritional immunology have been directed to the disease sequelae of nutritional deficiency or excess conditions, but there is some evidence

that high quality proteins (identified according to quantity, digestibility and the balance of amino acids and an ability to allow maximum growth) can differ in their capacity for supporting immunocompetence. Experiments on mice have shown that bovine whey proteins are superior to casein, several other animal proteins, several plant and algal proteins, in supporting antibody responses (Bounous and Gold, 1991; Wong and Watson, 1995). Whey proteins are a group of milk proteins that remain soluble in milk serum or whey after precipitation of caseins at pH 4.6 and temperature of 20°C. The major whey proteins in cow's milk are β-lactoglobulin, α-lactoglobulin, immunoglobulin, and serum albumin in the order of decreasing amounts (Eigel et al., 1984). A whey protein concentrate has been shown to be superior to other nutritionally equivalent diets containing an equal amount of casein in supporting the antibody based resistance of young adult mice to pneumococcal infection (Bounous and Kongshavn, 1989). Whey proteins can enhance adaptive immunocompetence when provided simply as a dietary supplement rather than as the sole protein source (Wong and Watson, 1995). These studies demonstrated that a change in the type of dietary protein can lead to an increased resistance to infection above normal without interfering with systemic nutrition.

The type of protein in the diet appears to influence the capacity of B lymphocytes to respond to an immunogenic stimulus. Whey proteins contain high levels of cysteine and their immunoenhancing activity has been attributed to an ability to support high levels of glutathione in lymphoid organs during response to antigenic challenge (Bounous and Gold, 1991). Another possibility is the upregulation of synthesis of the third component of complement, a protein considered important in the activation of B- lymphocytes by

macrophage from rodents consuming a whey protein based diet (Parker and Goodrum, 1990). However, Klasing and Barnes (1988) could not observe a consistent influence of immunogen injection on feed intake across two nutritionally complete diets in chickens. They observed that immunogen injected chicks fed corn isolated soybean protein based diets had significantly lower feed intake than saline injected chicks. However, immunogen injected chicks fed a corn, corn-gluten based diet had significantly increased feed intake. Although, feed intake was increased by immunogen injection, feed efficiency and weight gain were decreased in chicks fed corn-gluten based diet. This suggested a metabolic inefficiency in conversion of dietary protein and energy into body mass, as a result of the immunologic stress in *ad libitum* fed chicks (Klasing and Barnes, 1988). These studies indicate that the quality of proteins can differ in their capacity to support immune system.

2.1.3. Dietary factors that modify resistance to infection in general

According to Klasing (1998) dietary characteristics can modulate a bird's susceptibility to infectious challenges and subtle influences due to the level of nutrients or the type of ingredients at times of critical importance. Studies with experimental animals have shown that diet influences the overall composition of intestinal flora (Onderdonk, 1974). The diet may also have indirect regulatory effects that are mediated by the classical endocrine system. Physical and chemical aspects of the diet can modify the populations of microorganisms in the GI tract, the capacity of pathogens to attach to enterocytes, and the integrity of the intestinal epithelium.

Dietary factors that impact the microbial ecology of intestines include the amount of fiber, the viscosity of fiber, and fats that are refractory to digestion (Klasing, 1988). Barley

and rye which are high in water soluble nonstarch polysaccharides (NSPs) are associated with increased presence of *Clostridium perfringens* and increased incidence of necrotic enteritis (Hofshagen and Khaldhusdal, 1992; Riddle and Kong, 1992). Other dietary components besides fiber that influence the microbial ecology of intestines such as unstabilized rancid fat in the diet increases numbers of *E.coli* and lowers the number of *Lactobacillus* spp. (Dibner et al., 1996). Volatile fatty acids (VFA) produced by normally present harmless anaerobic bacterial flora in the ceca of chicken exert protective effects against multiplication of *Salmonella*. Couzin (1998) and Diez-Gonzalez (1998) have reported that feeding cows with hay may help prevent the multiplication of harmful strains of *E. coli* associated with hamburger disease, spread through beef.

2.1.4. Studies on the intervention of colonization of pathogenic organisms in the intestinal tract of birds

There have been a few research studies on specific intervention of colonization of /chickens by *Salmonella* and *Campylobacter*. Strategies such as the use of probiotics, prebiotics and synbiotics invariably target the manipulation of intestinal microflora to the advantage of the hosts.

Competitive exclusion (CE) is a concept introduced by Nurmi and Rantala (1973) for the purpose of reducing levels of *Salmonella* spp. in poultry. This approach provides for an undefined intestinal tract flora from specific pathogen free adult chickens and uses this source to furnish a bacterial subculture for treatment of newly hatched chickens. Concerns with this process are that poultry pathogens could potentially be transmitted through this undefined culture and that each CE culture is not equally effective in preventing colonization

by *Salmonella* spp. Humphrey et al. (1989) indicated that cecal contents from chickens inhibited *C. jejuni* in vitro. Soerjadi et al. (1985) indicated the applicability of subculture of fecal droppings from *Campylobacter-* free chickens. They reported that CE treatment reduced chicken colonization as monitored by cloacal swabs. But studies by Stern et al. (1988) and Shanker et al. (1990) could not observe any advantage in protecting chickens against *C. jejuni* with CE cultures.

Stern (1994) observed that mucosal competitive exclusion flora (MCE) was effective against both *Salmonella* and *C. jejuni* colonization. The Colonization Dose 50% (CD_{50}) was increased 100 fold in protecting against *C. jejuni* colonization. The mean $\log_{10}$ level of cecal carriage (colonization quotient) was similarly decreased by 100-1000 fold. But not all of the trials were successful in increasing the CD_{50} suggesting that all MCEs are not equal in effect.

Shoeni et al. (1992) observed that a nine strain mixture of cecal bacteria provided 41-85% protection from *C. jejuni* colonization. These strains included *Klebsiella pneumoniae* 23, *Citrobacter diversus* 22, and *E.coli* (O13H-) 25. Shoeni et al. (1994) found that defined competitive cultures and fructooligosaccharides (FOS) can be effectively administered to reduce *C. jejuni* colonization. Aho et al. (1992) showed that CE treatment may protect the birds against campylobacter but their studies revealed the need for facultatively anaerobic bacteria in establishing a protective flora. The results showed a 1.5 weeks' delay in the onset of campylobacter infection in treated chicks and consistently lower levels of colonization. Morishita et al. (1997) found that a commercial probiotic consisting of *Lactobacillus acidophilus* and *Streptococcus faecium* was able to reduce colonization and frequency of fecal shedding of *C. jejuni* to some extent in market aged broilers. Line et al. (1998) studied

the effect of yeast supplemented feed on *Salmonella* and *Campylobacter* populations. They observed that *Salmonella* colonization was significantly reduced but yeast treatment did not significantly affect *Campylobacter* colonization.

Feeding chickens lactose supplements increased the acidity of cecal contents and this inturn increased the amount of VFA which inhibited *Salmonella* (Corrier et al., 1991;1997). According to Hinton et al. (1991) treatment of a combination of cecal anaerobes and dietary lactose caused a greater reduction in the cecal colonization of *Salmonella typhimurium* and *E. coli* 0157: H7. Nisbet et al. (1993) and Corrier et al. (1993) have found that optimum protection against *Salmonella typhimurium* was obtained when birds were treated with continuous flow culture in combination with dietary lactose. Oyofo et al. (1989) have observed that mannose in drinking water could reduce colonization of *Salmonella* as D-mannose blocks *Salmonella* adherence to chicken intestine. Hume et al. (1993) have concluded that addition of propionic acid in the feed was ineffective in reducing *Salmonella* in the crop and ceca. Adding arabinose and galactose to the feed significantly reduced recovery of *Salmonella* (McHan et al.,1991). According to Bailey et al.(1991) Fructooligosaccharides (FOS) produced a four-fold reduction in the level of *Salmonella* in the ceca. They also noted that FOS in the diet may lead to a shift in the intestinal gut flora and under some circumstances may result in reduced susceptibility to *Salmonella* colonization.

Tellez et al. (1993) and McElroy et al. (1994) have found that continual dietary capsaicin administration increased resistance to *Salmonella enteritidis* colonization and organ invasion. Hanninen, (1990) has reported that the use of bismuth subsalicylate inhibited

the growth of campylobacters in a statistically significant manner in infant mice. According to Bovee-Oudenhoven et al. (1997) dietary calcium decreases the cytotoxicity of intestinal contents and intestinal epitheliolysis which not only strengthens the barrier function of the gut mucosa but also reinforces the protective, endogenous microflora.

However, the effects of feeds of different compositions on the colonization of *Campylobacter jejuni* in the intestinal tract of broiler chickens have not been subjected to a study so far. The hypothesis was that diet formulations containing proteins from different sources of origin would influence the intestinal colonization of *C. jejuni* in broiler chickens. The main objective of this study was to examine how feeds containing protein sources of animal origin and plant origin influenced the colonization of *C. jejuni* in the intestinal tract of broiler chickens.

The digestive system of birds is simple and hence their digestion is mainly enzymatic. Therefore highly digestible nutrients are a requirement. The cecum of a bird is a blind cul de sac with very little or no role in digestive processes. The *Campylobacter* spp. have selected the ceca as their specialized niche for colonization because the prevailing microenvironmental factors are favorable for their colonization and they do not encounter any disturbances there. When we include some poorly digestible dietary components of plant origin in poultry feed the ceca become active. The indigenous microbiota harboring the ceca of birds initiate a fermentation process which alters the physicochemical nature of the environment within the ceca (Klasing, 1998b). These changes in the environment favor the multiplication of certain microorganisms which at the same time is deleterious for the survival of certain other organisms. We could exploit this to the advantage of the host, so that

the colonization of certain pathogenic organisms harboring the ceca can be reduced. An important aspect in the difference between diets of animal and plant origin is their iron content. Diets containing animal proteins are rich in heme iron which can be readily utilized by the microorganisms for their needs. Availability of iron from feed ingredients of plant origin is very low as they are bound by certain compounds such as phytates and tannin. Low availability of iron is a limiting factor for the growth and colonization of microorganisms including *Campylobacter* in the gastrointestinal tract. Many organisms synthesize specific chelators called siderophores to bind iron in low iron environments. However, *Campylobacter* has few iron uptake systems in comparison to other organisms. The growth of *Campylobacter* is supported by the presence of heme iron in a low iron medium (Picket et al., 1992), whereas transferrin, lactoferrin and ferritin were found unable to provide iron to strains tested (Park and Richardson, 1995). *Campylobacter* organisms do not ferment or oxidize carbohydrates. When a diet is designed basically composed of components of plant origin, the availability of iron is reduced and also a favorable atmosphere congenial for fermentation processes in the ceca is created.

2.2. Materials and methods

2.2.1. Approval

This study was designed in accordance with the guidelines of the Canadian Council of Animal Care (CCAC, 1993) and was approved by the University of Prince Edward Island Animal Care Committee. Also, the approval of the Biohazards Safety Committee was obtained before the commencement of the experiment for handling *Campylobacter jejuni* organisms in biocontainment level II.

2.2.2. Isolation of a field strain of *C. jejuni* for infecting birds

2.2.2.1. Sample collection and processing

For isolation of a field strain of *C. jejuni*, ceca harvested from birds slaughtered in a Poultry Processing Plant located in Prince Edward Island (Waddell's, Crapaud) were used. Viscera of freshly killed birds (18) were collected immediately on paper trays during the evisceration process. The ceca were cut and tied and placed in sterile plastic stomacher bags (Fisherbrand, Canada) and kept on ice in an insulated cooler box and transported to the laboratory.

The cecal samples were transferred onto sterile petri dishes inside a biological containment hood. The surface of the cecum was cauterized with a red hot spatula and was cut open using a sterile scalpel blade. Swabs from cecal contents and mucosa were collected using a sterile cotton tipped applicator (Puritan, Hardwood Products, Maine, USA) and streaked onto Campylobacter Blood Free selective medium plates (CBF Oxoid, Canada; composition vide appendix B). The inoculated plates were incubated at 42° C for 48 hours under microaerophilic conditions. Campylobacter microaerophilic gas generating kit (Oxoid, Canada) was used in a GasPak jar (Oxoid, Canada) to provide the microaerophilic

conditions consisting of 5% Oxygen, 10% Carbon dioxide and 85% Nitrogen.

2.2.2.2. Identification of isolates

Colonies which appeared morphologically as small, raised or flat whitish grey with regular edges were gram stained. Those colonies which were gram negative slender curved rods were subcultured onto blood agar (BA, Oxoid, composition vide appendix B) plates and incubated at 42° C for 48 hours as described previously under microaerophilic conditions. The isolated colonies were characterized and confirmed as *Campylobacter jejuni* using the following tests; oxidase, catalase, hippurate, and latex agglutination test (JCL Kit, Integrated Diagnostics Inc, Baltimore, USA) and susceptibility to antibiotics, nalidixic acid (30 μg) and cephalothin (30 μg). The confirmed colonies were harvested and transferred into 1.5 mL sterile 2% skim milk taken in sterile screw cap vials (Cryovial) using sterile cotton swabs and kept frozen at -76° C.

2.2.3. Preparation and standardization of inoculum

The strain of *C. jejuni* selected (designated as AVC 13/1 and serotyped as *Campylobacter jejuni* subsp. *jejuni* Biotype IV HS O: 21, O: 29, HL untypeable) for inoculum preparation was thawed and grown microaerophilically on BA plates. Twenty four hour old colonies were harvested and transferred into 50 mL of Brain Heart Infusion (BHI, Oxoid, composition vide appendix B) in a sterile 150 mL Erlenmeyer flask and the optical density (OD) of the inoculum was measured using a spectrophotometer (Spectronic 20D, Milton Roy Company, USA) at 500 nm λ. Five hundred microliters of the inoculum was serially diluted in 4.5 mL BHI taken in Eppendorf tubes and a volume of 25 μL was spotted from each dilution on CBF and BA plates and incubated under microaerophilic conditions

for 48 hours for counting of colonies. The flask was capped loosely after placing a sterile magnet in it. The flask was kept in a Gas Pak jar (Campygen 3.5 L, Oxoid) along with a gas generating kit (Oxoid, Canada). The Gas Pak jar was kept on a magnetic stirrer and incubated for one hour with stirring at 37° C. After incubation, 10 mL of inoculum was diluted with nine volumes of BHI and the optical density was again measured. Five hundred microliters of the inoculum were serially diluted in 4.5 mL BHI (sequential 10 fold dilutions) and 25 μL portions were spotted over 10 spots from various dilutions and incubated as described earlier. After incubation the colonies were counted using a stereoscope illuminator (Wild M3Z, Heerbrugg, Switzerland) and the number of colony forming units/ mL (cfu/ mL) of the inoculum was calculated. These experiments were repeated seven times.

2.2.4. Pilot Study

A pilot study was undertaken to evaluate the colonizing potential of the strain selected for infecting birds used for the dietary study and to evaluate its recoverability. Three 16 day chick embryos were obtained from the Diagnostic Virology Laboratory of the Atlantic Veterinary College (AVC) and hatched in the Animal Care Facilities of AVC. The hatched chicks were moved into a cage, with pine wood shavings as litter in a room in the North Barn of AVC. They were identified using leg bands so that individual birds could be followed and sampled throughout the trial. The birds were fed for *ad libitum*, consumption a chick starter ration (Purina Start and Grow, 6039, Purina, Ontario, Canada; nutrient specification vide appendix A) and were provided with water sterilized by autoclaving.

2.2.4.1 Preparation of inoculum

An inoculum containing approximately 10^8 cfu / mL of *C. jejuni* was prepared as

described in section 2.2.3. On the third day two birds were orally gavaged with 0.5 mL of the inoculum prepared using a tuberculin syringe.

To recover the bacteria from the birds, cloacal swabs were taken using calcium alginate swabs (Calgiswab, Type 4, Hardwood Products Comp, Maine, USA) the swabs were rotated five times inside the cloaca five times as described by Morishita et al. (1997), and Achen et al. (1998). The swabs were immediately plated onto CBF plates and incubated microaerophilically for 48 hours.

After incubation the colonies grown were gram stained and those colonies which appeared as gram negative slender curved rods were subcultured onto BA plates and incubated microaerophilically for 24 hours. The colonies grown were harvested and transferred into 1.5 mL, sterile skim milk (2%) taken in screw capped vials (Cryovial) and stored frozen at -76° C for further characterization. This routine was continued until day 13 of the experiment. On the 13th day after taking cloacal swabs the birds were euthanized with carbon dioxide and necropsied. The ceca, jejuni and crops were harvested for quantitation of bacteria to determine the carriage of *C. jejuni* in the respective gastrointestinal segments.

2.2.4.2. Quantitation of *C. jejuni*

Empty sterile stomacher bags 7.5cm x 17.5cm (Fisherbrand, Canada) were weighed and specimens were transferred into the stomacher bags. The weights of bags plus the specimen were recorded and then 0.1% peptone water (composition vide appendix B) was added at the rate of nine times the weight of the specimen and homogenized for one minute using a stomacher (Stomacher Lab blender 400, Seward Medical, London, UK). One

hundred microliters of the homogenized suspension were transferred into 900 μL of 0.1% peptone water. By transferring 100 μL from the first dilution to the second, second to third and so forth, sequential 10- fold dilutions were made. A duplicate dilution was also prepared in the same way. Twenty five microliters of each dilution were spotted on ten spots on CBF plates and kept for drying. Following drying the plates were incubated microaerophilically for 48 hours. After incubation, the colonies developed were counted using a stereoscopic illuminator (Wild M3Z, Heerbrugg, Switzerland). Colonies typical of *C. jejuni* were counted on 10 spots from plates. Their mean value was used to calculate the number of organisms in the original sample by the application of appropriate dilution factor. Thus, the cfu/g of the organ cultured for both replicates were determined and their mean value was recorded as the cfu /g of that particular organ.

2.2.5. Main study

2.2.5.1. Birds and diet

In this trial broiler birds were used. The period of study was for 35 days, corresponding to the current broiler production cycle. Hatching eggs were purchased from Clark's Chick Hatchery (Moncton, New Brunswick), reported as collected from a flock with an excellent health record, the health status of which had been constantly monitored. The flock was vaccinated regularly for all diseases prevalent in the region and those vaccinations were backed up by periodic testing. The eggs, on arrival from the hatchery were disinfected by dipping in 2% formaldehyde for five minutes. After drying, 180 eggs were set at 37.5° C in a disinfected incubator in the Animal Care Facilities of the AVC for hatching.

Three different diet formulations were designed for the study and consisted of ingredients containing protein sources from 1. Animal origin 2. Plant origin and 3. A combination of both plant and animal origin. The diets were prepared by Co-operative Atlantic, Agricultural Division (Moncton, New Brunswick) and supplied as mash feed in 20 Kg bags. The diet formulations and nutrient specifications are given in Tables 2.1 and 2.2. The mixed feed comprising of a combination of both plant and animal proteins, was prepared by mixing equal quantities of feeds of animal and plant origin. The feeds are called animal, plant and mixed hereafter.

Table 2.1. Diet formulations

Name of ingredients	Percentage		
	Animal protein based	Plant protein based	Mixed protein based
Ground corn	57.8	47.3	52.55
Ground wheat	20.6	12.3	16.45
Meat meal	6.0	-	3.0
Poultry by- products meal	6.0	-	3.0
Fish meal	6.0	-	3.0
Feather meal	3.0	-	1.5
Soybean meal	-	20.0	10.0
Canola meal	-	5.0	2.5
Corn gluten meal	-	6.0	3.0
Poultry grease	-	4.3	2.15
Phosphate	-	2.3	1.15
Salt	0.5	0.5	0.5
Calcium granules	-	2.0	1.0
Methionine	0.07	0.05	0.06
Lysine	0.05	-	0.025
T. B. Mineral mixture[1]	0.05	0.05	0.05
Broiler Vitamin mix[2]	0.1	0.1	0.1
Poultry mineral mixture[3]	0.05	0.05	0.05

[1] indicates turkey broiler mineral mixture. Provides in % - iron- 4.4, manganese- 12.2, zinc- 11.0, copper- 0.9, iodine- 0.1 and in mg/kg, selenium- 300.

[2] Broiler vitamin mixture; provides (in iu/kg)- Vit A- 10 000 000, vit D -3000 000, vit E- 30 000, menadione- 2000 mg/ kg, vit B_{12}- 15000 mcg/ kg, riboflavin- 7000 mg/ kg, niacin -50000mg/kg, pantotheinc acid- 15000, threionine- 1500, pyridoxine- 3000, biotin- 200, folic acid- 1000.

[3] Poultry mineral mixture; provides, in %- iron -3.0, manganese- 6.6, zinc- 7.0, copper- 1.0, iodine- 0.1, and selenium- 300 mg/kg.

Table 2.2. Nutrient specification of diets.

Name	Units	Amount		
		Animal protein	Plant protein	Mixed Protein
Weight	Kg	1.000	1.000	1.000
M. E. Poultry	Kcal/ Kg	3162.2	3160.9	3161.6
Crude fiber	%	2.7	2.9	2.8
Crude fat	%	4.7	6.9	5.8
Crude protein	%	20.1	20.0	20.05
Methionine	%	0.44	0.44	0.44
T. S. A. A*	%	0.78	0.79	0.785
Lysine	%	0.91	0.92	0.915
Calcium	%	1.22	1.23	1.225
Total Phosphate	%	0.84	0.84	0.84
Available Phosphorus	%	0.70	0.60	0.65
Sodium	%	0.24	0.20	0.22
Linoleic acid	%	1.41	1.68	1.545
Xanthophyll	Mg/ Kg	12.7	28.0	20.35
Choline	Mg/ Kg	1153.0	1224.9	1188.95
Vitamin A	IU/ Kg	10000.0	9995.0	9997.5
Vitamin D	IU/ Kg	3000.0	2998.5	2999.25
Vitamin E	IU/ Kg	30.0	29.98	29.99

Asterisk (*) indicates Total sulfur amino acids

2.2.5.2. Experimental design and layout

The control and experimental birds were housed in separate rooms which had been throughly cleaned and disinfected. The rooms selected were located about 50m apart from each other in order to minimize the possibility of transmission of infection from the treatment group to the control birds.

Inside each room, six floor pens were set up to house the birds belonging to each feed group. Each pen was approximately 1.65m x 0.86m x 0.73m size and was made of polyvinyl chloride (PVC) 2.5cm diameter pipe and covered with wire mesh of 2.5cm opening. Hanging brooders (Can Arm, Ontario) fitted with infrared heat lamps (Phillips: 250W) were used in each pen to provide supplemental heating according to requirements outlined in CCAC (1993) guidelines. The litter used consisted of pine wood shavings which was tested in advance to ensure freedom from *Campylobacter*. Feeders (5.4 Kg poultry feeder) and waterers (4.5 L) were purchased new and were thoroughly washed, cleaned and disinfected before use.

One hundred and twelve chicks were randomly allocated to 14 groups, each group consisting of eight birds. Six groups were designated as the controls and another six as the experimental. The two remaining groups were necropsied at the start to ascertain that the birds used in the study were free of *Campylobacter* and *Salmonella*. Each dietary treatment was replicated in two separate pens with 8 chicks per pen (total 16 chicks per diet), in both control and experimental groups. Computer software (Quattro Pro, Corel Corporation, Canada) was used for randomization protocols.

2.2.5.3. Animal husbandry and management

The birds were individually weighed and identified using leg bands. They were provided with feed and water *ad libitum*. The water was sterilized by autoclaving. Litter was placed in the pens approximately seven cm deep. The temperature and humidity in each pen were checked and adjusted periodically. The temperature in the pen was checked and height of the heat lamps adjusted to provide the optimum heat. The leg bands were replaced with bigger sized ones before they became tight on their legs.

Strict biosecurity protocols were observed while attending the daily routines of feeding, providing water and sample collection. Disposable coveralls (Tyvek, Dupont), gloves (Fisherbrand, Canada), footwear (Fisherbrand, Canada), and disinfectant footbath were used every time entry was made into either rooms. The litter was changed once every week. The coveralls, gloves, footwear and used litter were disposed off by incineration. Access to the facilities was strictly restricted.

2.2.5.4. Testing of environment and feed for *Campylobacter* and *Salmonella*

Air, litter and feed were sampled and cultured to ensure that birds in the control group did not carry any *Salmonella* and *Campylobacter*, nor were infected from the environment and that the study group did not get any *Campylobacter* other than the inoculum. Litter samples and feed samples were obtained by pooling approximately one gram quantities. Air was tested for *Campylobacter* before housing the birds using the settle plate method (Collins et al., 1992) by culturing six CBF plates after keeping open in various locations in each room for 48 hours. The litter and feed samples were cultured for *Salmonella* and *Campylobacter* organisms by enrichment procedures. An enrichment broth for isolation of *Campylobacter*

was prepared (Doyle and Roman, 1982; composition vide appendix B).

Thirty grams each of ground feed and litter samples were placed in 100 ml of the enrichment broth. The samples were incubated with vigorous agitation under microaerophilic conditions at 42° C for 18 hours. Small quantities of the supernatant were then plated onto CBF plates and incubated microaerophilically at 42° C for 48 hours for detection of growth of *Campylobacter*.

For testing *Salmonella*, the feed and litter samples were placed in Rappaport broth (Oxoid; composition vide appendix B) and incubated for 72 hours at 42° C under vigorous agitation and aliquotes from the supernatant were plated onto Modified Semisolid Rappaport- Vassiliadis (MSRV, Oxoid; composition vide appendix B) plates onto 4 spots and incubated for another 48 hours at 42° C in a walk in incubator.

2.2.5.5. Testing of birds for *Campylobacter* and *Salmonella*

To make sure that the birds used in the study were free of *Campylobacter* and *Salmonella*, the 16 birds randomly selected were euthanized on the first day of experiment, using carbon dioxide and their ceca, jejuni and crop were harvested. Swabs were taken from the specimens using sterile cotton tipped applicators (Puritan, Maine, USA) and were plated onto CBF plates and incubated microaerophilically at 42° C for 48 hours. For detecting *Salmonella*, swabs were placed in Rappaport broth for enrichment and incubated for 72 hours at 42° C and then plated onto MSRV plates and incubated for 48 hours.

2.2.5.6. Exposure method

The strain of *Campylobacter jejuni* which was shown to be capable of colonizing the birds in the pilot study was used for infecting the birds. An inoculum containing approximately 10^8 *Campylobacter jejuni*/ mL was prepared as described in section 2.2.4. Cloacal swabs were taken from all the birds in both experimental and control groups prior to the administration of inoculum and plated onto CBF plates. All the birds in the experimental group were administered orally with 0.5 mL of the inoculum using tuberculin syringes at three days of age.

2.2.5.7. Sample collection for recovery of *C. jejuni*

Urethrogenital calcium alginate swabs (Calgiswab type 4, Hardwood Products Comp, Maine, USA) were used to collect cloacal samples from birds. Collection of samples was made first from birds in the control group and then from the experimental group. The cloacal swabs were collected as described and immediately plated onto CBF plates. The plates were then incubated under microaerophilic conditions for 48 hours in a Gas Pak jar maintained at 42° C. The colonies grown were subcultured onto BA plates after confirming morphology by gram staining. The colonies grown were transferred into sterile skim milk and stored at -76° C for further studies. The sampling was done once every three days until the end of the experiment.

2.2.5.8. Euthanasia, necropsy and sample collection for quantitation of *C. jejuni*

On the last day of the experiment (day 35), after taking cloacal swabs, all birds were weighed and then euthanized using carbon dioxide. After recording their weights, 4 birds at a time were put in a basin with a lid at a time and carbon dioxide gas was passed from a

cylinder for 5 minutes. The birds were necropsied after disinfecting their lower abdomen by spraying with 70% isopropanol. Their ceca, jejuni and crop were harvested and stored frozen for quantitation of *C. jejuni*.

2.2.6. Characterization of isolates

The frozen isolates were thawed, and were transferred onto BA plates using sterile cotton tipped applicators (Puritan, Hardwood Products, Maine, USA) and incubated under microaerophilic conditions at 42° C for 48 hours. The resulting colonies were characterized by the following tests; oxidase, catalase, hippurate, latex agglutination test and susceptibility to nalidixic acid and cephalothin.

2.2.6.1. Oxidase test

A few colonies of the isolate were placed on a piece of tissue paper and two drops of oxidase reagent (BBL oxidase, Becton Dickinson, USA) were dropped over them. Development of a purple color indicated a positive reaction.

2.2.6.2. Catalase test

A few colonies were placed on a clean glass slide and hydrogen peroxide (3%) was dropped over it. A positive reaction was indicated by the formation of bubbles which were visualized under a stereoscope illuminator (Wild M3Z, Heerbrugg, Switzerland).

2.2.6.3. Hippurate test

Two to three colonies of the isolate were emulsified in 100 μL of sterile distilled water taken in a plastic tube and a rapid hippurate disk (Remel, Lenexa, Kansas, USA) was placed in it and incubated for 2 hours at 37° C. Following incubation, 2 drops of ninhydrin reagent (Remel, Lenexa, Kansas, USA) were added, mixed thoroughly and again incubated

for 30 minutes. Development of blue to purple color indicated positive reaction.

2.2.6.4. Latex agglutination test

Index campy (JCL, Integrated Diagnostics Inc, Baltimore, USA) was used for the test. The test kit components consisted of a test slide with embossed circles, extraction reagent, neutralization reagent, positive control reagent and latex detection reagent. The reagents were warmed to room temperature and one free falling drop of the reagent, was dispensed onto each specimen circle and the negative control circle. Using a wooden applicator stick, an isolated colony was removed from each isolate and a homogenous suspension was made by rotating the inoculum containing stick in the extraction reagent. Then, one drop of the neutralization reagent was dispensed into each specimen circle and the negative control circle. One drop of the positive control reagent was dropped into the positive control circle. One drop of latex detection reagent was dispensed on each circle on the slide and mixed well with wooden sticks. The slide was placed on a platform shaker (Orbit Shaker, Labline Instruments, Inc, Ill., USA) and rotated for 5 minutes at room temperature. The reaction was observed under a stereoscope illuminator (Wild M3Z, Heerbrugg, Switzerland). A positive reaction was indicated by the development of agglutination, except in the circle of the negative control.

2.2.6.5. Antibiotic sensitivity tests

A few colonies were transferred into 500 μL deionized distilled water in a round bottom polystyrene tube 12 x 75 mm (Falcon. Becton Dickinson) and adjusted to a turbidity of 0.5 McFarland standard by comparing with standard turbidity tubes (Oxoid, Canada). A sterile cotton tipped applicator 15 cm (Puritan) was dipped in the suspension and excess

fluid was removed by pressing and rotating the swab firmly against the sides of the tube. The swab was then streaked evenly in three directions on the entire surface of a Mueller Hinton-Blood Agar (MH-BA, Oxoid, composition vide appendix B) plate to obtain a uniform lawn of bacteria. The plates were dried for 15 minutes and then using a disk dispenser (Oxoid, Canada), antibiotic paper disks (nalidixic acid and cephalothin) were placed on the plate. After leaving for 10 minutes to dry the plates were incubated microaerophilically in a jar with gas pack at 42° C for 48 hours. Following incubation the plates were examined and the zones of inhibition (diameter in mm) were measured using a ruler and recorded.

2.2.6.6. Biotyping and serotyping of isolates

The isolates selected for biotyping and serotyping were sent to the National Laboratory for Enteric Pathogens (NLEP), Canadian Science -Center for Human and Animal Health, Winnipeg, Manitoba, Canada. The frozen cultures were thawed and the organisms were grown on BA plates and the colonies were harvested using sterile cotton tipped applicators and kept in brucella transport medium with blood and packed in SAF- T- Pak observing all regulatory requirements and forwarded by courier service to Winnipeg.

2.2.7. Quantitation of *Campylobacter jejuni*

The ceca, jejuni and crops of birds stored frozen were thawed and the number of *C. jejuni* was determined as described previously in section 2.2.4. Randomly selected colonies were tested and confirmed as *C. jejuni* using standard methods of identification as previously described.

2.3. Results

2.3.1. Isolation of a field strain of *C. jejuni*

Growth of moist, spreading or discrete colonies resembling *C. jejuni* was observed on all CBF plates cultured. The typical colonies morphologically suggestive of *C. jejuni* were gram stained and those colonies which showed gram negative organisms with characteristic slender, curved rod shape were subcultured onto BA plates and incubated under microaerophilic conditions at 42° C for 24 hours.

2.3.2. Characterization of isolates

The results of the various phenotypic tests are shown in Table 2.3. All isolates appeared as gram negative curved slender rods on gram staining. They were positive for catalase, oxidase and latex agglutination tests. Also, they were sensitive to nalidixic acid (30 μg) and resistant to cephalothin (30 μg). The species differentiation into *Campylobacter jejuni* and *Campylobacter coli* was made on the basis of hippurate hydrolysis test. Out of 17 isolates tested 16 were hippurate positive, while one was hippurate negative. About 62.5% of isolates gave intense reaction as indicated by the color to the hippurate hydrolysis test and the rest gave moderate reaction. Those isolates which gave a positive reaction to the hippurate test were classified as *Campylobacter jejuni* and the one which was negative was classified as *Campylobacter coli*. Most of the isolates were found to form discrete individual colonies while a few, formed spreading colonies. The isolate # 8 (vide Table 2.3) which formed spreading colonies was selected for infecting birds in the study.

Table 2. 3. Results of various phenotypical tests to identify isolates.

Isolate #	Gram staining	Colony type	Catalase	Oxidase	Hippurate	Latex test	Antibiotic sensitivity	
							NA 30	CE 30
1	gram negative curved rods	D	+	+	+	+	+	-
2		D	+	+	+	+	+	-
3		D	+	+	++	+	+	-
4		D	+	+	++	+	+	-
5	„	S	+	+	++	+	+	-
6	„	D	+	+	+	+	+	-
7	„	D	+	+	+	+	+	-
8*	„	S	+	+	++	+	+	-
9	„	D	+	+	++	+	+	-
10	„	D	+	+	++	+	+	-
11	„	D	+	+	++	+	+	-
12	„	D	+	+	++	+	+	-
13	„	D	+	+	++	+	+	-
14	„	S	+	+	++	+	+	-
15	„	D	+	+	++	+	+	-
16	„	D	+	+	-	+	+	-
17	„	D	+	+	+	+	+	-

Legends: + positive reaction; ++ intense reaction; - negative reaction, D- discrete colonies; S- spreadng colonies; NA 30- nalidixic acid 30 μg; CE 30- cephalothin 30 μg; * the strain selected for infecting chickens.

2.3.3. Dose standardization of inoculum

A standardized dose of *C. jejuni* was required to infect the birds. The number of cfu was correlated with OD so that inoculum with known cfu could be administered to the birds. An OD of 0.025-0.035 approximately corresponded to 10^8 cfu/mL of *C. jejuni*. The results of the various trials on dose standardization are given in Table 2.4.

2.3.4. Pilot study

The strain used for inoculating the birds in the pilot study was found to readily colonize the birds. Cloacal swab cultured yielded *C. jejuni* organisms from chicks # 1 and #3 on all days from the second day of inoculation. Chick #1 showed high intensity of shedding, chick #3, moderate shedding while chick # 2 remained infection free until day 12. The *C. jejuni* load (cfu/g) of the various intestinal organs as determined by quantitation is given in Table 2.5. All three birds were found to be colonized with *C. jejuni* in their ceca and jejuni while only one bird (#1) carried organisms in its crop.

2.3.5. Main Study

2.3.5.1. Testing of environment and feed for *Campylobacter* and *Salmonella*

The plates cultured to detect the growth of *Campylobacter* and *Salmonella* using the respective enrichment procedures did not show growth of any of these organisms. This showed that the environment and feed did not carry any *Campylobacter* and *Salmonella* that could serve as a potential source for extraneous infection of the birds used in the study.

2.3.5.2. Testing of birds for *Campylobacter* and *Salmonella*

The ceca, jejuni and crops harvested and cultured on the first day of the experiment from all 16 randomly selected birds were negative for both *Campylobacter* and *Salmonella*.

Table 2. 4. Approximate correlation between optical density and cfu/ mL of *C. jejuni* inoculum.

# of trial	Optical density (nm)	counts cfu/ mL ($\log_{10}$)
1	0.062	8.301
2	0.046	8.778
3	0.062	8.255
4	0.036	8.653
5	0.031	8.301
6	0.021	8.579
7	0.045	8.681

Table 2. 5. Isolation of *C. jejuni* from intestinal organs quantitatively cultured.

Intestinal segment	Organisms cfu/g (log_{10})		
	chick # 1	chick # 2	chick # 3
Cecum	6.812	4.174	4.488
Jejunum	3.893	2.845	3.346
Crop	2.602	-	-

2.3.5.3. Infection of birds with *C. jejuni*

No *C. jejuni* was isolated from birds prior to challenge. The challenge inoculum contained approximately 10^8 cfu/ mL of *C. jejuni* organisms and oral inoculation of birds did not elicit any untoward reactions. The necropsy results of birds died during the course of the study are given in appendix C.

2.3.5.4. Recovery of *C. jejuni*

Most birds (mean of 83.3%) excreted *C. jejuni* within 24 hours of inoculation. As fecal shedding reflects intestinal colonization, the growth of *C. jejuni* was used as a criterion for determining the shedding pattern of the organism. Shedding of *C. jejuni* from birds fed different diets is given in Table 2.6. On the day following infection (day 4) birds were found excreting organisms from the various groups at the following rates; the mixed feed group, 87.5%, animal feed group, 87.5% and plant feed group, 75.0 % with an overall of 83.3%. On day 7 the percentage rates were 75.0, 87.5, 85.7 respectively for mixed, animal and plant feed groups with an overall rate of 82.6 %.On the day of necropsy (day 35) the shedding rates were 78.5, 85.7 and 66.6 for the mixed, animal and plant feed groups respectively with an overall of 77.5 %. Except for days 16 and 31, the rate of shedding was less for plant feed group compared to animal feed group.

To determine whether there was any significant difference between the shedding of *C. jejuni* in birds fed various diet formulations the data were analyzed using oneway Analysis of variance. The model evaluated three feed levels against shedding percentage. Although on an overall basis there was no significant difference ($p > 0.05$) in the shedding pattern of organisms among birds fed different diets, the shedding rate on the day of

necropsy was found to be the lowest in the plant feed group suggesting a lower colonization in birds fed a plant protein based diet. The graphical representation of the shedding pattern from day 4 to day 35, created using computer software (Minitab™ Version 12 for windows) is given in Fig 2.1. The graph is suggestive of a rising and falling pattern of shedding among all birds irrespective of the feed group they belonged.

2.3.5.5. Performance of chickens

To determine whether there was any significant difference between the performance of birds the weights of the birds in various feed groups, were analyzed using the General linear model procedure (SAS Institute, Inc. 1995). The model evaluated three feed levels against weights of the birds. There was no significant difference in weight gain of birds ($p > 0.05$) in the various feed groups. The weight gain of birds fed different diets in the experimental group are given in Table 2.7. On average the birds gained 0.05 Kg per day in all three feed groups. On the last day of experiment (day 35) the birds in the mixed feed group (n = 14) had a mean weight of 1.74 Kg with Standard Error of Mean (SEM) of 0.48. The mean weight of birds of the plant feed group (n = 12) was 1.71 Kg with SEM of 0.05 and that of the animal feed group (n = 14) was 1.76 Kg with SEM of 0.46.

2.3.5.6. Characterization of isolates

The results of characterization of isolates are given in Tables 2.8.1 and 2.8.2. All isolates were gram negative slender curved rods morphologically resembling *C. jejuni* was positive for oxidase, catalase hippurate and latex agglutination tests. On disk sensitivity testing to antibiotics nalidixic acid (30 μg) and cephalothin (30 μg), all isolates were found sensitive to nalidixic acid and resistant to cephalothin (30 μg). The isolates were confirmed

as *Campylobacter jejuni* using the standard tests previously outlined.

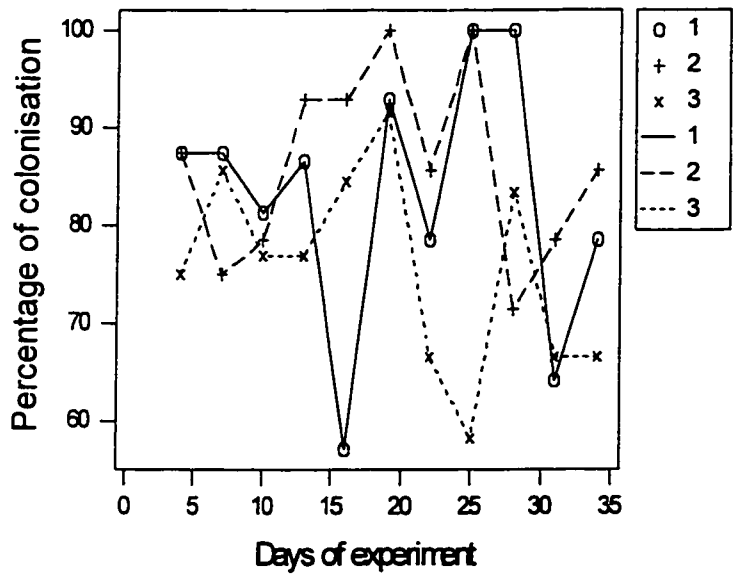
2.3.5.7. Biotyping and serotyping

Results of biotyping and serotyping with heat labile-Lior (HL) and heat stable-Penner (O) schemes of isolates are given in appendix D and a summary is given in Tables 2.9. The strain of *C. jejuni* used for infecting chicken used in this study and 95% of isolates recovered were reported to belong to biotype IV, Penner (HS) serotype HS O: 21, : O: 29. Whereas 5% of the isolates were biotype II and serotype HS O: 2. According to the Lior (HL) scheme the strain inoculated and 75% of the isolates recovered were reported as HL untypeable, while about 20% were classified as HL 40 while 5 % to HL 4.

Table 2. 6. Shedding of *C. jejuni* by challenged birds fed different diets.

Days	% of birds shedding			
	Mixed	Animal	Plant	Overall
4	87.5	87.5	75.0	83.3
7	75.0	87.5	85.7	82.6
10	78.5	81.2	76.9	79.0
13	92.8	86.6	76.9	85.7
16	92.8	57.1	84.6	78.0
19	100.0	92.8	91.6	95.0
22	85.7	78.5	66.6	77.5
25	100.0	100.0	58.3	85.0
28	71.4	100.0	83.3	85.0
31	78.5	64.2	66.6	70.0
34	85.7	78.5	66.6	77.5
35	78.5	85.7	66.6	77.5

Fig 2.1. Shedding pattern of *C. jejuni* from day 4 to day 35.



Legends: feed groups: 1, mixed; 2, animal; and 3, plant. Continuous line (__), mixed; broken line (---), animal; dotted line (.....) plant.

Table 2.7. Performance of birds fed different diets over 35 day

variables	feed groups									*p* value
	mixed			plant			animal			
	n*	mean	SEM	n*	mean	SEM	n*	mean	SEM	
	uninfected birds									
weight(Kg)										
initial	16	0.067	0.001	16	0.071	0.001	16	0.067	0.001	0.08
final	13	1.650	0.070	15	1.69	0.030	16	1.750	0.040	0.12
weight gain		1.583			1.619			1.683		
average weight gain (Kg/day)		0.045			0.046			0.048		
	infected birds									
weight(Kg)										
initial	16	0.068	0.001	16	0.066	0.001	16	0.068	0.001	0.15
final	14	1.750	0.480	12	1.710	0.100	14	1.750	0.460	0.74
weight gain		1.681			1.644			1.682		
average weight gain (Kg/day)		0.048			0.047			0.048		

Legends: asterisk (*) indicates # of birds, SEM indicates Standard Error of Mean.

Table 2.8.1. Characterization of isolates recovered from birds fed different diets.

# of isolate	Gram staining	Oxidase test	Catalase test	Hippurate test	Latex agglutination test	Sensitivity to antibiotics	
						NA30	KF30
M1	negative	+	+	+	+	+	-
M2	slender	+	+	+	+	+	-
M3	curved	+	+	+	+	+	-
M4	rods	+	+	+	+	+	-
M5	„	+	+	+	+	+	-
M6	„	+	+	+	+	+	-
M7	„	+	+	+	+	+	-
M8	„	+	+	+	+	+	-
M9	„	+	+	+	+	+	-
M10	„	+	+	+	+	+	-
M11	„	+	+	+	+	+	-
M12	„	+	+	+	+	+	-
M13	„	+	+	+	+	+	-
M14	„	+	+	+	+	+	-
P1	„	+	+	+	+	+	-
P2	„	+	+	+	+	+	-
P3	„	+	+	+	+	+	-
P4	„	+	+	+	+	+	-
P5	„	+	+	+	+	+	-
P6	„	+	+	+	+	+	-
P7	„	+	+	+	+	+	-
P8	„	+	+	+	+	+	-
P9	„	+	+	+	+	+	-
P10	„	+	+	+	+	+	-
P11	„	+	+	+	+	+	-
P12	„	+	+	+	+	+	-

Abbreviations: M: mixed feed group; P: plant feed group; +: positive reaction; - : negative reaction; NA: 30 nalidixic acid 30 μg; KF 30: cephalothin 30 μg.

Table 2.8.2. Characterization of isolates recovered from birds fed different diets (contd.).

# of isolate	Gram staining	Oxidase test	Catalase test	Hippurate test	Latex agglutination test	Sensitivity to antibiotics	
						NA30	KF30
A1	negative	+	+	+	+	+	-
A2	slender	+	+	+	+	+	-
A3	curved	+	+	+	+	+	-
A4	rods	+	+	+	+	+	-
A5	„	+	+	+	+	+	-
A6	„	+	+	+	+	+	-
A7	„	+	+	+	+	+	-
A8	„	+	+	+	+	+	-
A9	„	+	+	+	+	+	-
A10	„	+	+	+	+	+	-
A11	„	+	+	+	+	+	-
A12	„	+	+	+	+	+	-
A13	„	+	+	+	+	+	-
A14	„	+	+	+	+	+	-

Abbreviations: A: animal protein feed; +: positive reaction; - : negative reaction; NA 30: nalidixic acid 30 μg; KF 30: cephalothin 30 μg.

Table 2.9. Summary of results of biotyping and serotyping.

Scheme	Classification	% of isolates
Biotype	IV	94.87
	II	5.12
Serotype		
HS (Penner)	O:21 O: 29	94.87
	O: 2	5.12
HL (Lior)	UT	74.35
	4	5.12
	40	20.51

Legends: HS: heat stable, HL heat labile UT untypeable

2.3.5.8. Quantitation of *C. jejuni*

The ceca of birds fed plant based diet appeared enlarged visually in comparison to those of birds in the mixed and the animal feed groups. The ceca, jejuni and crops from birds belonging to control (n = 44) and treatment (n = 40) groups were quantitatively cultured. In total 252 specimens were cultured from both treatment and control groups. *C. jejuni* did not grow on any plates cultured quantitatively with organs harvested from birds fed different diets in the control group. The growth of organisms was observed on plates cultured with organs harvested from birds belonging to the various treatment groups. *C. jejuni* were recovered from the cecum of all birds (100%) fed different diets. The isolation rates from jejuni of birds were at the following rates; mixed feed group (92.8%), animal feed group (92.8%) and for plant feed group (83.3%). Organisms were isolated from the crops of birds belonging to the three treatment groups in the following rates; 78.5%, 50.0% and 41.6% from mixed, animal and plant feed groups respectively.

The results of quantitative culturing of various organs are given in Tables 2.11- 2.13. The colonization, cfu/ g ($\log_{10}$) of ceca of birds belonging to mixed feed group ranged from 5.21 to 6.55. In the case of birds belonging to animal feed group, the cfu/ g ($\log_{10}$) of ceca, the highest value was 6.86 and the lowest value 5.71. Whereas, in the birds belonging to plant feed group the cfu/ g ($\log_{10}$) of ceca the values ranged from 3.72 to 6.69. The corresponding values for jejuni of birds belonging to mixed feed group, the highest value being 4.19 and lowest being 2.60. In the birds belonging to animal feed groups the cfu/ g ($\log_{10}$) of jejuni the values ranged from 2.68 to 4.83. For the birds in the plant feed group the cfu/ g ($\log_{10}$) of jejuni, the highest value being 3.80 and lowest 2.60. The cfu/ g ($\log_{10}$) of

crop of birds belonging to mixed feed group the value ranged from 2.60 to 3.63. The corresponding mean value of birds belonging to animal feed group the highest value of 3.02 and lowest being 2.68. In the case of birds belonging to plant feed group the cfu/ g ($\log_{10}$) value ranged from 2.60 to 3.28.

To determine if there was any significant difference in the intestinal colonization of *C. jejuni* in three feed groups the data were analyzed by a Mixed model procedure (SAS Institute, Inc.,1995).The statistical model evaluated the classes (organ, feed and bird) in three levels against colonization (cfu/g). The tests of fixed effects were on feed, organ and organ-feed. Residual components were analyzed using the Univariate procedures (SAS Institute, Inc., 1995) for skewness, kurtosis and normality. The results are shown in Table 2.14. The mean colonization of *C. jejuni* cfu/ g ($\log_{10}$) in the ceca of birds in the mixed, plant and animal feed groups were 5.87, 4.98 and 6.39 respectively. The corresponding values for jejuni were 3.29, 2.89 and 3.47 respectively. The values for crops were 3.08, 2.95 and 2.82 in the respective feed groups. The Least Square Mean values of colonization cfu/ g ($\log_{10}$) of various organs in the feed groups which showed significant differences were graphed (Fig 2.2). From the data analysis it was evident that the effect of feed was not independent but was dependent on the organ cultured. The effect due to differences in feed was significant in the case of the cecal colonization of *C. jejuni* but the effects were nonsignificant in the cases of jejunum and crop. The birds fed plant based diet had the lowest cecal colonization of *C. jejuni* in comparison to other feeds.

Table 2.11. Colonization [cfu/ g (log_{10})] of *C. jejuni* in cecum of individual challenged birds (n = 40) fed different diets.

cfu/ g (log_{10}) of ceca of birds of feed groups		
Mixed (n = 14)	Animal (n = 14)	Plant (n = 12)
5.944	6.580	5.761
6.551	6.863	3.934
6.245	6.466	5.716
6.227	6.269	6.696
5.568	6.436	5.225
6.290	6.629	3.723
6.086	6.536	5.298
5.929	5.878	4.120
5.450	5.716	3.748
5.463	6.521	5.748
5.392	6.480	5.025
6.017	5.713	4.795
5.968	6.698	
5.209	6.681	

Legends: Mixed: birds fed mixed diet; Animal: birds fed animal protein based diet and Plant: birds fed plant protein based diet. cfu/g; colony forming units/g.

Table 2.12. Colonization [cfu/g (log_{10})] of jejunum of individual birds (n = 40) fed different diets.

cfu/ g (log_{10}) of jejuni bird of feed groups		
Mixed (n = 14)*	Animal (n = 14)*	Plant (n = 12)*
3.716	4.012	2.924
3.816	3.682	2.954
4.190	4.837	3.807
4.136	3.235	2.949
4.068	4.041	2.653
3.851	3.056	2.949
3.993	2.684	2.690
2.602	3.217	2.602
2.949	3.096	2.662
3.060	2.785	2.681
2.740	2.977	
2.676	3.913	
3.000	3.068	

Legends: Mixed: birds belonging to mixed protein based diet; Animal: birds belonging to animal protein based diet and Plant: birds belonging to plant protein based diet. cfu/g; colony forming units/ g. * organisms were recovered from 13 birds each and ** from 10 only.

Table 2.13. Colonization [cfu/g (log_{10})] of crop of individual birds (n = 40) fed different diets.

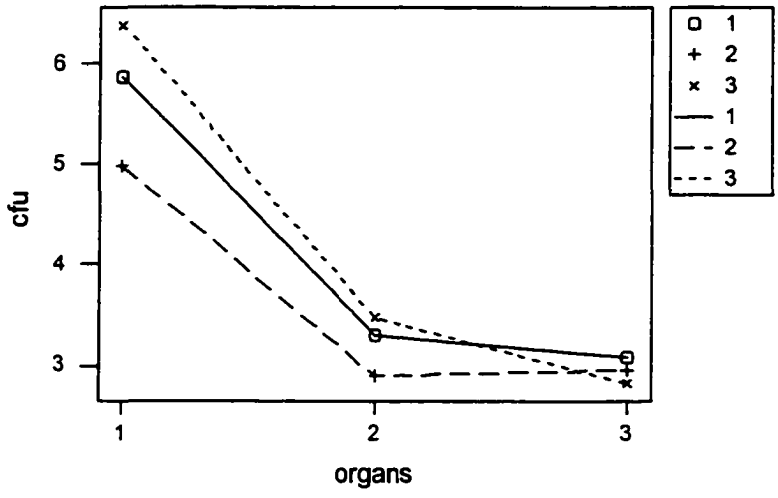
cfu/ g (log_{10}) of crop of birds feed groups		
Mixed (n = 14)*	Animal (n = 14)**	Plant (n = 12)***
3.636	3.021	3.283
3.260	2.681	2.767
3.257	2.688	2.997
3.605	2.726	3.214
3.041	2.977	2.602
3.851	2.949	
2.602	3.017	
2.946		
2.698		
2.748		
2.602		

.Legends: Mixed: birds belonging to mixed protein based diet; Animal: birds belonging to animal protein based diet and Plant: birds belonging to plant protein based diet. cfu/g; colony forming units/ g. * organisms isolated from 11 birds, ** organisms recovered from 7 birds and *** 5 birds only.

Table 2.14. Analysis of cfu/ g of *C. jejuni* in various organs of birds (n = 40) fed different diets.

Feed group	Organ	Colonization cfu/g (log_{10})		Confidence Interval (95%)	T value	Pr >T
		LS Mean	SE			
	Cecum	5.8797	0.1496	5.5864-6.1730	39.3	0.001
Mixed	Jejunum	3.2953	0.1554	2.9907-3.6000	21.2	0.001
(n = 14)	Crop	3.0876	0.1691	2.7561-3.4192	18.3	0.001
	Cecum	4.9828	0.1562	4.6766-5.2890	31.9	0.001
Plant	Jejunum	2.8932	0.1701	2.5598-3.2266	17.0	0.001
(n = 12)	Crop	2.9516	0.236	2.4888-3.4143	12.5	0.001
	Cecum	6.3909	0.1446	6.1074-6.6743	44.2	0.001
Animal	Jejunum	3.4771	0.1556	3.1721-3.7821	22.3	0.001
(n = 14)	Crop	2.8228	0.2002	2.4303-3.2153	14.1	0.001

Fig. 2.2. Comparison of mean cfu/g of colonization (log_{10}) of *C. jejuni* in different gastrointestinal organs of birds fed different diets.



Legends: On the X axis, 1 represents the organ, cecum; 2 jejunum and 3 crop. On the Y axis cfu represents the colonization of *C. jejuni* expressed in colony forming units/g (log_{10}). Continuous line (—) represents mixed feed; broken line (— —) plant feed and dotted line (.....) animal feed.

2.4. Discussion

2.4.1. Isolation and identification of *Campylobacter*

Although *Campylobacter* are ubiquitous and present in large numbers in the intestinal tracts of birds and other animals the isolation of these organisms poses certain practical difficulties. It requires special media and incubation conditions. The selective media used for isolation should contain a combination of antimicrobial agents to which *Campylobacter* spp. are resistant along with agents to suppress the growth of yeasts at correct proportions. Problems encountered in the isolation process were (1) the overgrowth of swarming colonies of proteus spp. and (2) spreading of *Campylobacter* on the plate without forming individual colonies. It was found useful to observe the plates for the growth of *Campylobacter* immediately after incubation for 24 hours, to prevent the swarming growth of proteus spp. that may interfere with the isolation. Spreading of colonies was due to the motility of the organism in the presence of moisture and this was prevented by incubating the plates at 37^0 C for a couple of hours before plating to reduce the moisture. In a previous study the prevalence of *Campylobacter* in chickens slaughtered in Prince Edward Island was determined to be around 51.5% (Ahmed et al., 1996). They have also reported that the *C. jejuni* was the predominant species, with a rate of about 85% and the rest *C. coli.*

Characteristics such as growth at higher incubation temperature, cultural, morphological and staining characteristics, immunological tests and sensitivity to antibiotics were used to identify *Campylobacter* spp. The test used for species differentiation in *Campylobacter* is hippurate hydrolysis; those positive are designated as *C. jejuni* and those negative as *C. coli.*

2.4.2. Pilot study

The purpose of the pilot study was to ascertain the colonization potential of the strain used to inoculate the birds. It was observed that the strain selected readily colonized the GI tract of birds as evidenced from the fecal shedding. This was important because some noncolonizing strains of *C. jejuni* also exist. The pilot study was needed to determine the onset and duration of shedding following a single oral inoculation of *C. jejuni*. It was possible to recover organisms beginning the following day of challenge and subsequent days until the end of the trial from two birds which were orally gavaged. The shedding pattern observed was continuous. The birds tolerated the challenge dose of the organisms very well and did not exhibit any ill effects or diarrhea after the procedure. Cohabitation of a noninfected bird along with infected birds produced fecal shedding and colonization after about 12 days. Although the same dose was administered to both birds, the intensity of shedding was found to be high in one bird and moderate in the other bird. The intensity of shedding may correlate well with the degree of intestinal colonization. This was also evident in the results of the quantitative culturing of organisms following necropsy. Therefore, the degree of fecal shedding of the organism as determined by cloacal swab culturing may serve as a noninvasive method to ascertain the degree of intestinal colonization in live birds. The highest degree of colonization among intestinal segments cultured was observed in the cecum, followed by the jejunum and organisms were recovered from the crop of one bird as well.

2.4.3. Main study

The purpose behind the main study was to determine how feeds with different protein sources influence the colonization of *C. jejuni* in the intestinal tract of birds. The testing of

feed, litter and air using enrichment procedures did not yield any *Campylobacter* or *Salmonella*. *Campylobacter* are not found normally in fresh feed or the hatchery environment unlike *Salmonella* for which feed may serve as a potential source of infection (Stern, 1992). Old litter can serve as a source of *C. jejuni* organisms (Genigeorgis et al., 1986) but usually fresh litter is free of them (Montrose et al., 1985). The results of testing of chicks randomly selected for *Campylobacter* and *Salmonella* were also negative. This ensured that all birds used in the study were free of these organisms and did not interfere with challenge with *C. jejuni*.

Recent studies suggested that vertical transmission through contaminated eggs may play a role in the transmission of *Campylobacter* as it does with *Salmonella enteritidis* in poultry (Cox et al., 1999 and Pearson et al., 1996). Therefore, it was necessary to make sure that the chicks used in the study were free of *C. jejuni*. The predominant source of *C. jejuni* infection in the farm is through the water supply (Pearson et al., 1993). Sterilization of water by autoclaving ensured that the birds did not get *Campylobacter* from drinking water. *Campylobacter* are usually acquired during the second or third week of their life through contact with environmental fomites. To prevent cross contamination of the organism from the infected birds to the control birds, strict biosecurity protocols were observed. No *C. jejuni* was recovered from the cloacal swabs and the intestinal organs of the control birds suggesting that adherence to good sanitary protocols and strict biosecurity measures could effectively prevent the colonization of these organisms in birds. But this is not very practicable in farming situations.

The field strain of *C. jejuni* found capable of colonizing birds without producing any

adverse health effects in the pilot study was used for the main study also. A few mortalities observed during the course of the trial. These occurred sporadically and were within accepted limits. The mortalities were subjected to a detailed necropsy conducted in the post mortem room of the AVC. Findings of the necropsy were incidental (vide appendix C).

The weight gain in birds fed the three different diets were not significantly different. This assured that all birds in all feed groups grew equally well and hence differences in nutritional status of any birds in the different feed groups did not influence the colonization of these organisms.

Mucus colonizing organisms generally show a tendency to adhere to alginate and so the use of calcium alginate swabs for the recovery of *C. jejuni* from the cloaca of birds was found useful. *C. jejuni* were not isolated before experimental infection of the birds. This provided additional evidence that the birds used in the study were free of these organisms. The inoculum prepared contained approximately 10^8cfu/ mL and each bird was given 0.5 mL of the inoculum. A challenge dose of 0.5 mL of an inoculum containing10^8 cfu/ bird was found adequate for colonization in this study and agreed with previous studies by other investigators who had found that chicks 1- 3 days old were usually colonized by *C. jejuni* when challenged with 10^4 -10^8 cfu (Sanyal et al., 1984 and Stern et al., 1988). However, Schoeni and Doyle, (1992) observed that 8- 9 day old chicks needed 10^8 - 10^{10} cfu for adequate colonization. The reason for this could be that colonization of chicks may be affected by many factors and perhaps older birds may need more organisms for colonization. Following infection, it was possible to recover organisms from birds 24 hours after infection and about 83.3% of birds were found shedding organisms in their cloaca. Birds in the control

group did not excrete any organisms suggesting that these birds did not harbor any *C. jejuni*. Establishment of organisms in the intestinal tract results in extensive fecal excretion (Welkos, 1984), so fecal shedding and recovery of organisms from the cloaca indirectly reflect the intestinal colonization. But all birds did not excrete the organisms during each sample collection uniformly or at the same rate. A rising and falling pattern was observed in the fecal shedding of organisms. This agreed with the report of Achen et al. (1998) who suggested a cyclicity in their shedding. The exact reason for this pattern is not clearly understood. Although there was no significant difference in the shedding of organisms in different diet groups, the percentage of birds shedding *C. jejuni* was found to be less in birds fed plant protein based feed toward the marketing age of birds. This observation may be significant because this reduction reflects reduced intestinal colonization which means reduced risk for carcass contamination.

The isolates recovered from birds belonging to different feed groups were characterized by various tests such as gram staining, oxidase, catalase, hippurate, latex agglutination and sensitivity to antibiotics and were confirmed as *Campylobacter jejuni*. The isolates were subjected to biotyping and serotyping at a national reference laboratory for enteric pathogens. The challenge strain and 95% of isolates recovered from the birds were reported as biotype IV serotypes HS O: 21, O: 29. But 5% of isolates recovered belonged to biotype II serotype HS O: 2. According to the Lior (HL) scheme the strain used for challenge and 75% of isolates recovered were reported as HL untypeable, 5% to HL 4 and 20% to HL 40. According to Hanninen et al. (1999) genotypic and phenotypic changes including change in serotypes occur after passage of *C. jejuni* through chicken intestine. About 95% of isolates

were found identical to the strain used for challenge, according to the biotyping and Penner Heat Stable serotyping schemes. The reason for the isolates being untypeable in the HL scheme is not known. One possible reason may be the incomplete coverage of all existing serotypes by the HL (Lior) scheme. *Campylobacter* serotypes are not species specific; some types have been found in both *C. jejuni* and *C. coli*. The influence of environmental factors on the stability of most of these phenotypic properties is a major weakness of these techniques (Yan et al., 1991). Phenotypic techniques have a high nontypeable rate in veterinary isolates (Newell and Wagenar, 2000). By identifying both the HS and HL antigens, the discriminating potential between different strains could be achieved unlike when a single antigen is identified (Hood et al., 1988). Combination testing allows recognition of heterogeneity among strains colonizing chickens (Sjogren and Kaijser, 1989).

It was possible to recover *C. jejuni* from the cecum of all birds belonging to all feed groups. The colonization [cfu/ g ($\log_{10}$)] of ceca ranged from 4.9 to 6.3. The cecum has been described as the major site of colonization of *C. jejuni*. The highest colonization observed being in the animal feed group and the lowest in the plant feed group. The data on cecal colonization agreed with previous reports which ranged from 4.0 $\log_{10}$ to 7.0 $\log_{10}$ (Doyle, 1991 and Achen et al., 1998). The colonization (cfu/ g) ranged from 2.8 to 3.4 ($\log_{10}$) in the case of jejuni of birds belonging to the three feed groups, the highest being in the animal feed group and lowest in plant feed group.

Byrd et al. (1998) have reported that they could isolate *Campylobacter* from the crops of 7 week old birds at a significantly different frequency than from the ceca. They studied the effects of feed withdrawal prior to processing of broiler birds on the recovery of

campylobacters from the crop and found that there was a significant ($p < 0.025$) increase in the contamination of crops in birds withdrawn from feed than those fullfed. In another study, the average counts of *C. jejuni* in the crop of birds reported were 2.9 x 10^3 cfu/ g (Achen et al., 1998). However, no information is available on how these organisms get into the crop of birds. In this trial it was observed that higher number of *C. jejuni* could be recovered from the crops of birds. The mean cfu/ g ($\log_{10}$) of crop of birds in the different feed groups ranged from 2.8 to 3.1, the highest being in the mixed feed group and lowest in the animal feed group. Therefore, further studies are needed to ascertain whether this is an external contamination (hungry birds with a voracious appetite ingesting fecal matter or litter when feed is withdrawn) or an actual colonization. This is important because the potential for carcass contamination from crops is as high as that of ceca due to their rupture during processing.

2.4.3.1. Diet and colonization

The approach taken in this study was to combine the information available on the unique features of *Campylobacter* colonization in the intestinal tract of birds with our current knowledge of digestion in birds and effects of dietary factors on the colonization of microorganisms, in order to exploit these to our advantage to reduce the colonization of these organisms in the intestinal tract of broiler birds.

Campylobacter are unique in certain respects with regard to their colonization of the intestinal tract of birds. They are ecologically adapted to the avian GI tract and have selected a special niche as a safe haven for colonization where the microenvironmental factors are favorable for their survival and multiplication. The temperature of birds is conducive for

thermophilic campylobacters. They do not ferment or oxidize carbohydrates. The microenvironmental conditions in the ceca are congenial to their microaerophilic requirements of growth. The emptying of cecal contents is slow as peristaltic activity is very much reduced. They colonize the cecal crypt mucus without attaching to the microvilli (Beery et al., 1988). These organisms are chemotactically attracted to L- fucose, a component of mucin and they can utilize mucin as a sole substrate for growth (Hughdahl et al., 1988).

The indigenous microflora in the intestinal tract of birds play an important role in the metabolism of gut lumen and colonization of pathogens (Savage, 1986). The response of an animal to an infection depends on its immune status while the immune status is dependent on its nutritional status. Diet appears to influence, along with other factors, resistance to infection because specific nutrients are important in endocrine regulation, immune and somatic cell function, antibody production and tissue integrity. Especially, micronutrient and antioxidant interactions may increase disease resistance through regulating cellular and molecular processes, including membrane integrity and flux, superoxide formation and leukocyte function (Tengerdy et al., 1983). In addition to dietary factors strengthening the immune function and contributing to disease resistance, physicochemical and compositional factors of diet also influence the growth and/or colonization of microorganisms in the intestinal tract. These factors include temperature, pH, water activity, OR potential, nutrient content and presence of certain inhibitory substances. The interaction between diet and microorganisms may be beneficial to the host at some times but harmful at other times. Some organisms such as *E. coli* or *Clostridium difficile* present in small quantities may overgrow at favorable circumstances such as prolonged treatment with antibiotics, or drastic changes

in feed compositions may produce disease. The microbial dynamics of the intestinal tract is influenced by many dietary factors. Diet is the substrate, and hence the characteristics of its components become crucial. The diet or substrate determines what organism is to be favored or disfavored. If one substrate is growth limiting and if two organisms are limited by the same substrate, only one will be favored and the other organism will be forced to divide at a slower pace (Hansen and Hubbel, 1980). Also, the population of microorganisms, the species of the host and the microenvironmental conditions that prevail in the gastrointestinal tract are significant. The microbial ecosystem of the gastrointestinal tract is very complex, composed of hundreds of species, most of them either harmless or beneficial while a few may be pathogenic. Intestinal microflora acquired during earlier stages of life is very much dependent on feeding. Once a flora is acquired and stabilized, very little compositional changes occur subsequent to advancing age. Few population of organisms are affected by radical changes in diet but the overall composition remains stable over time.

In the case of birds, the main substrate that affects the microflora in the intestinal tract is the carbohydrates and this effect is dependent upon the type of carbohydrate. The review of literature suggested that water soluble carbohydrates might increase microbial activity. In birds, although the digestion is mainly autoenzymatic, a little alloenzymatic digestion also takes place due to microbial fermentation in the ceca. Fermentation of nondigestible dietary substrates is a major metabolic function of microflora (Roberfroid et al., 1995). The products of fermentation are gases such as hydrogen and carbon dioxide, short chain fatty acids (SCFA), lactic acid, ethanol, branched chain fatty acids (BCFA), ammonia, amines, phenols and indoles (Roberfroid, et al., 1995). Gases are the main products of microbial metabolism. Depletion of oxygen or accumulation of carbon dioxide could change OR potential and pH.

Short chain fatty acids are the metabolic end points of carbohydrate fermentation while the BCFA result from the oxidative breakdown of amino acids. They also play a major role in metabolizing primary bile acids thus participating in enterohepatic recycling and in the metabolism of cholesterol (Roberfroid et al., 1995). The pH of luminal contents become more acidic as a result of production of carboxylic acids.

The main aim behind this study was to ascertain how diets that differ in protein sources influence the colonization of *C. jejuni* in the intestinal tract of birds. The quantitation of *C. jejuni* (cfu/ g) from organs cultured showed that the diets used in this study had an organ dependent significant interaction on the colonization of *C. jejuni* in the intestinal tract of birds. The difference in colonization observed in the ceca is important because the rupture of that organ contributes much to the contamination of the meat during processing with *C. jejuni*. A reduction in the cecal load, therefore, would reduce the risk of carcass contamination. The differences observed in the colonization of *C. jejuni* in birds fed with various combinations of diet could probably be explained as the effect due to a combined interaction of various substrates in the diet. Although the birds have a simple digestive system, a little fermentation also takes place in their ceca where the microenvironmental conditions are favorable for the process.

One observation made in the present study about the relative sizes of the ceca of birds in the different feed groups, was that the ceca of birds in the plant protein group was visually the largest, followed by mixed protein group and then the animal protein group. This could probably be an adaptation to accommodate the fermentation that took place in the cecum. When certain components such as water soluble non starch polysaccharides are present in the diet they may favor the fermentation process giving rise to products such as lactic acid, VFA

and gases. These components would probably alter the physicochemical conditions such as the temperature, pH, and OR potential, thereby, creating a microenvironment within the cecum that may have inhibitory effects on many organisms. A lowering of pH or increase in acidity is detrimental to a wide range of organisms (Corrier et al., 1990; Chambers et al., 1997). The changes in OR potential also affects composition of microorganisms (Sinell, 1980). Reduced OR potential favors facultative anaerobes and anaerobiosis slows the growth of facultative organisms. The gases produced as well as the lowered pH as a result of fermentation may have increased the OR potential (Sinell, 1980). High concentration of VFA have inhibitory effect on many organisms (Nisbet et al., 1993). Each of these factors, pH, water activity, OR potential may significantly influence microflora and many of these factors are interdependent. So it is desirable to take into account the total ecology of the food.

In the diet formulations used in this study the content of soluble NSPs in the animal protein based diet was 0.92% against 3.6% in the plant protein based diet (calculation vide appendix E). It means that the presence of sNSPs in the plant based diet might have contributed considerably significantly to enhance microbial fermentation in the GI tract of chickes fed with plant based diet in comparison to other diet formulations.

Iron is an essential substrate for *C. jejuni* and changes in the availability of iron could affect their colonization. Animal origin diets are rich in heme iron with a better bioavailability in comparison to plant origin foods (Walter, 1997) and this may contribute to the growth of many microorganisms including *C. jejuni*. On the other hand, iron present in plant based diets are found in a bound form (Freeland- Graves, 1988). The presence of dietary fiber, phytate and tannin further reduces its bioavailability (Bindra and Gibson, 1986). In addition, diets high in soyprotein reduce nonheme iron bioavialability (Cook et al., 1981). All these factors

would contribute to reducing the availability of iron to microorganisms. The mineral supplements used in the diet formulations were added at the same level and so provided the same iron content to all feed formulations. However, the iron content of the animal protein based diet was higher than plant protein based diet (vide calculation in appendix E). Moreover, the iron present in plant based feeds is found in a bound form and hence its bioavailability is low in comparison to iron found in animal based feeds. A low iron environment is favorable for the growth of lactobacilli which, not only do not require iron for their growth but also are inhibited in a high iron environment. Therefore a plant based diet might have favored the growth of lactobacilli which produced lactic acid and contributed to the suppression of other organisms. Combined interactions of all the above factors together might have contributed to the observed reduction in the cecal load of *C. jejuni* in birds fed the plant protein based diet.

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3. ANTIMICROBIAL SUSCEPTIBILITY PATTERNS

3.1. Background

The emergence of resistance to antimicrobial agents has compromised the treatment of infections due to many bacterial pathogens and has become a concern for both human and animal health. The development of resistance may result from direct use of antimicrobial agents or acquisition of resistance factors from animal or environmental flora of animals that have been exposed to antimicrobial agents. The purpose of this study was to determine whether there were any significant differences in the susceptibility patterns of *C. jejuni* isolates to antimicrobial drugs, which could be helpful for detecting introduction of a new strain or changes in patterns due to the effects of feed. Whether factors such as changes in diet composition influence on emergence of resistant strains are not known and hence, the determination of the susceptibility of isolates to different antimicrobial agents is important to the theme of the present study.

The objectives of this study were to determine

1. Whether or not antimicrobial resistance has developed during the in vivo experiment and if so the identification of antimicrobial agents to which the isolates have become resistant.
2. Use of resistance patterns as an epidemiological tool to identify various isolates from the experiment.

Acquisition of resistance usually occurs following a chromosomal mutation or acquisition of foreign DNA through plasmid or transposon (Taylor and Courvalin, 1988). All *C. jejuni* isolates are intrinsically resistant to bacitracin, novobiocin, rifampin, streptogramin B, trimethoprim, vancomycin, and cephalothin (Taylor and Courvalin, 1988). *Campylobacter*

spp. were originally described as highly susceptible to erythromycin, fluoroquinolones, tetracyclines, aminoglycosides, clindamycin and moderately susceptible to chloramphenicol, cefotaxime, ceftazidime and cefpirome (McNulty, 1987). However, resistance to various other antibiotics has been reported. *Campylobacter* have acquired resistance to tetracycline and ampicillin (Brooks et al., 1986, Taylor and Courvalin, 1988). Resistance to erythromycin and kanamycin is also on the rise (Karmali et al., 1981, Papadopoulou and Courvalin, 1988). Altwegg et al. (1987) have shown evidence for *Campylobacter* acquiring resistance to nalidixic acid and norfloxacin in a patient who was under adequate treatment with norfloxacine. High level cross resistance occurs in campylobacters to quinolones including ciprofloxacin, norfloxacin, and tamofloxacin (Gootz and Martin, 1991). Resistance to tetracycline and kanamycin is plasmid mediated (Taylor and Courvalin, 1988). Erythromycin resistant campylobacters were also found resistant to tylosin, spiramycin, and clindamycin (Burridge et al., 1986). Resistance to macrolides, lincosamide and streptogramin appears to be chromosomally determined (Taylor and Courvalin, 1988). Resistance to quinolones may involve the DNA gyrase enzyme itself or could be due to the inability of the drug to penetrate (Taylor and Courvalin, 1988). Campylobacter organisms may transfer resistance to other campylobacter strains by conjugation (Tenver and Elvrum, 1988). They may also acquire resistance determinants from gram positive organisms (Papadopoulou and Courvalin, 1988). So it is desirable to track down the emergence of resistance in campylobacters and the possible mechanisms of acquisition of resistance to various antibiotics commonly used.

Susceptibility testing is usually done by either dilution or diffusion methods. The diffusion methods such as disks and E-test strips have been used commonly. The E-test (PDM

Epsilometer, AB Biodisk, Solna, Sweden) has been found to compare favorably with agar dilution methods for most antibiotics (Baker, 1992). Interpretation of susceptibility results when testing campylobacter is most often difficult due to the lack of standards for testing methods and accepted breakpoints for determining resistance. For this reason, statistical tests were used to evaluate the likely differences in antimicrobial sensitivity profiles of isolates in this study.

3.2. Materials and methods

3.2.1. Disk diffusion test

To evaluate the antimicrobial susceptibility profile of isolates, Kirby-Bauer disk diffusion testing was performed using 12 antibiotics. The antibiotics used in the study and their concentration are given in parenthesis. [Ciprofloxazin (CIP 30μg), Nalidixic acid (NA 30μg), Tetracycline (TE 30μg), Amikacin (AK 30 μg), Gentamicin (CN 10μg), Streptomycin (S 10μg), Chloramphenicol (C30μg), Enrofloxacin (ENR 5μg), Erythromycin (E 15μg), Kanamycin (K 30 μg), Ampicillin (AMP 10 μg), Cephalothin (KF 30μg)]. The antibiotic disks were purchased from Oxoid, Canada. Mueller-Hinton blood agar (MH- BA, Oxoid, composition vide appendix A) was used for the testing. Isolates recovered from birds in the dietary study described in Chapter 2 were randomly selected and used in this study.

Twenty four hour old colonies grown on BA plates incubated microaerophilically were harvested and suspended in 0.5 mL deionized distilled water taken in 17 x 77mm polystyrene tubes (Falcon, Becton Dickinson, USA). The turbidity of the suspension was adjusted to 0.5 McFarland by comparing with standard turbidity tubes (Oxoid, Canada). A sterile cotton tipped applicator was dipped into the standardized culture suspension, and excess fluid was removed by pressing and rotating the swab firmly against the side of the tube just above the fluid level. The swab was then streaked evenly in three directions over the entire surface of the MH- BA plates in order to make a uniform lawn of bacteria. After leaving the inoculated plates to dry for 15 minutes antibiotic disks were applied on the plates using a mechanical disk dispenser (Oxoid, Canada). The plates were inverted and incubated at 42° C in an anaerobic jar under microaerophilic conditions. After 48 hours of incubation the plates were

examined and the zones of inhibition on the agar (diameter in mm) surface were measured using a ruler. For controls *Escherichia coli* ATCC strain 25922 and *Campylobacter jejuni* ATCC strain 33291 were tested in parallel to monitor the potency of the disks.

3.2.2. E-test for minimum inhibitory concentration (MIC) determination

E-test (PDM epsilometer, AB Biodisk, Solna, Sweden) consists of a non porous plastic strip calibrated with MIC values covering 15 two fold dilutions. A predefined antibiotic gradient is immobilized on the surface opposite the MIC scale. The following antibiotics, Ampicillin (AM), Kanamycin (KM), Chloramphenicol (CL), Streptomycin (SM), Gentamicin (GM), Tetracycline (TC), Ciprofloxacin (CI), Erythromycin (EM) were used in this study.

The E-test was performed according to the instructions of the manufacturer. The plates were prepared and incubated as described in section 3.2.1. E-test strips were applied on the plates using a forceps. After 48 hours of incubation the plates were examined and the zones of inhibition on the agar surfaces were read against the scale where the ellipse intersected the scale in accordance with the manufacturer's instructions. For controls *Escherichia coli* ATCC strain 25922, *S. aureus* ATCC 25923 and *Campylobacter jejuni* ATCC strain 33291 were tested in parallel.

3.3. Data analysis

To determine whether the antimicrobial susceptibility profiles of isolates from birds fed different diet formulations, were significantly different one way analysis of variance (one way ANOVA) was used (Minitab™ Inc).The model evaluated feed groups in three levels against the zones of inhibition of isolates. Significance was assumed at the 0.05 level.

3.4. Results

3.4.1. Disk diffusion method

Results of the antimicrobial susceptibility tests are presented in Tables 3.1.1 and 3.1.2. The summary of values of strain used (AVC 13/1) for infecting birds, isolates and control strains is furnished in Table 3.2. The strain used for infecting the birds was found to be resistant to tetracycline. All the isolates from the three treatment groups were also found to be resistant to tetracycline. All the tested isolates were found susceptible to nalidixic acid and resistant to cephalothin.

Data analysis employing a one way ANOVA revealed no significant difference between the zone of inhibition (diameter in mm) of isolates belonging to the three feed groups (p value > 0.05) for all the antibiotics tested.

Table 3.1.1. Mean zone of inhibition (mm) of antimicrobial agents with *C. jejuni* isolates from birds fed different diets.

Parameter	n	Mean zone of inhibition (mm) of antimicrobial agents				
		CIP 30	NA 30	AK30	CN10	S30
Feed groups						
mixed	15	35.13	27.13	31.33	30.73	29.33
plant	12	34.72	27.63	30.09	30.18	29.36
animal	16	36.00	28.56	32.43	33.18	29.81
pooled mean		35.11	28.04	31.28	31.63	29.50
pooled SEM		0.578	0.892	1.020	1.026	0.211
p value		0.322*	0.758*	0.288*	0.083*	0.215*

Abbreviations: CIP, ciprofloxacin; NA, nalidixic acid; TE, tetracycline; AK, amikacin; CN, gentamicin; S, streptomycin.* indicates nonsignificant results ($p > 0.05$). SEM standard error of mean.

Table 3.1.2. Mean zone of inhibition (mm)of antimicrobial agents with *C. jejuni* isolates from birds fed different diets (contd.).

Parameter	n	Mean zone of inhibition (mm) of antimicrobial agents				
		C 30	ENR 30	E 15	KA30	AMP10
Feed groups						
mixed	15	35.00	35.46	32.80	32.66	16.86
plant	12	35.63	36.09	34.45	31.09	17.27
animal	16	36.06	36.87	34.43	32.50	17.56
pooled mean		35.56	36.14	33.89	32.08	17.23
pooled SEM		0.565	0.726	0.621	0.708	0.539
p value		0.409*	0.346*	0.097*	0.264*	0.620*

Abbreviations: C, chloramphenicol; ENR, enrofloxacine; E,erythromycin; KA, kanamycin; AMP, ampicillin; KF, cephalothin.* indicates nonsignificant results ($p > 0.05$).SEM, standard error of mean.

Table 3.2. Summary of mean zone of inhibitions (diameter in mm) of 12 antibiotics for the strain used for inoculation (n = 3), the isolates (n = 42) and control strains, *C. jejuni* ATCC 33291(n = 5) and *E. coli* ATCC 25922 (n = 5).

Strain	Mean zone of inhibition (diameter in mm) of antimicrobial agents											
tested	CIP	NA	TE	AK	CN	S	C	ENR	E	K	AMP	KF
AVC												
13/ 1*	33	25	R	25	25	28	35	32	28	30	17	R
isolates	35	28	R	31	31	29	35	36	33	32	17	R
C. jejuni												
ATCC	35	25	34	30	30	26	30	36	34	25	15	R
33291												
E. coli	30	23	20	21	22	15	21	31	R	24	16	16
25922												

Abbreviations: CIP, ciprofloxacin; NA, nalidixic acid; TE, tetracycline; AK, amikacin; CN, gentamicin; S, streptomycin; C, chloramphenicol; ENR, enrofloxacin; E, erythromycin; K, kanamycin; AMP, ampicillin; KF, cephalothin; R, resistant. * strain used for inoculation of birds.

3.4.2. E-test

The results of E-test for 8 antimicrobial agents tested are given in Table 3.4. The summary of MIC values of strain infected, isolates and control strains are furnished in Table 3.5. All the isolates were found to be sensitive to all antibiotics tested except tetracycline to which all of them were found resistant. The MIC of erythromycin may be classified as intermediate as per the National Committee on Clinical Laboratory Standards (NCCLS) guideline breakpoints. In the analysis of data of MIC (μg/mL) of isolates using one way ANOVA there was no significant difference between isolates belonging to the different feed groups.

Table 3.3. Minimum inhibitory concentrations (μg/mL) of antimicrobial agents of *C. jejuni* isolates from birds fed different diets.

Measure	n	Minimum inhibitory concentration (MIC μg/L)						
		AM	EM	CI	GM	CL	KM	SM
FEED GROUPS								
mixed	14	2.0	0.857	0.09	0.484	0.714	4.0	0.808
plant	12	2.0	0.896	0.09	0.450	0.646	4.0	0.812
animal	14	2.0	0.857	0.09	0.414	0.661	4.0	0.771
pooled mean		2.0	0.869	0.09	0.450	0.675	4.0	0.797
pooled SEM		*	0.020	*	0.017	0.025	*	0.027
p value		*	0.686	*	0.231	0.529	*	0.541

Abbreviations: AM ampicilin; EM erythromycin; CI ciprofloxacin; GM gentamicin; CL chloramphenicol; KM kanamycin; SM streptomycin.* no SEM or *p* value as all values are equal.

Table 3.4. Summary of MIC (μg/mL) values determined using E- test of antimicrobial agents with *C. jejuni* ; strain used for challenge, the isolates recovered from birds and control strains.

Strains tested	Mean MIC (μg/mL) of Antimicrobial agents							
	AM	EM	CI	TC	GM	CL	KM	SM
AVC 13/ 1* Isolates	2	0.868	0.094	R	0.449	0.675	4	0.79
C. jejuni[1] 33291	3	1	0.38	0.1	0.25	2	4	0.38
E. coli[2] 25922	4	48	0.01	2	1	2	3	3
S. aureus[3] ATCC 25923	0.047	0.25	0.19	0.75	0.38	4	1.5	2

Abbreviations: AM, ampicillin; EM, erythromycin; CI, ciprofloxacin; TC, tetracycline; GM, gentamicin; CL. chloramphenicol; KM, kanamycin; SM, streptomycin; R, resistant.* strain used for inoculation of birds (n = 3), Control strains are marked with superscripts.

C. jejuni [1] (n = 4), *E. coli* [2] (n = 4), *S. aureus* [3] (n = 4).

3.5. Discussion

3.5.1. Disk diffusion method

The objective of this study was to determine the antimicrobial susceptibility of isolates to various antimicrobial agents and whether any change occurs in the antimicrobial susceptibility pattern of isolates from three different feed groups. All the isolates in the three feed groups were found sensitive to all drugs tested except tetracycline and cephalothin. The strain of *C. jejuni* used for inoculating the birds was also found to be resistant to these antimicrobial drugs. *Campylobacter* are naturally resistant to cephalothin but the resistance to tetracycline appears to be an acquired one. There are five different resistance genes to tetracycline and MIC varies considerably depending on the gene present. The treatments did not alter the antimicrobial sensitivity profile of drugs tested. In the data analysis of zone of inhibition (diameter in mm) from the susceptibility testing of the isolates in the three treatment groups there was no significant differences between the feed groups ($p > 0.05$). This meant that mean value of zones of inhibition of isolates in three feed groups did not differ in their antimicrobial susceptibilities to the drugs tested. In other words, changes in feed composition did not have an effect on the antimicrobial susceptibilities of the drugs tested in the study. In conclusion, in the present study changes in feed composition did not influence the antimicrobial susceptibility patterns of *C. jejuni* isolates.

3.5.2. E-test and MIC

The antimicrobial susceptibility profile of drugs used for E-test appeared pretty consistent in all isolates irrespective of their treatment groups. The average MIC of erythromycin of isolates was found to be 0.86 μg/mL which was slightly higher than that of NCCLS guidelines breakpoint (0.5 μg/mL) categorized as sensitive. According to Baker (1992) category interpretation of erythromycin results was a problem using the current NCCLS guideline breakpoints. The E-test and the agar dilution method tend to give similar results. According to Tenover et al. (1992) the MICs of erythromycin and tetracycline were slightly higher with E-test than with broth microdilution. This difference was due to the use of NCCLS breakpoints that do not correspond to the doubling dilution scheme used by the E-test. In the present study data analysis of MIC values showed no significant differences between the antimicrobial susceptibility profile of drugs tested in the different feed groups which supports the findings of the disk sensitivity profile.

Most of the commercially available methods of antimicrobial susceptibility testing do not perform well with campylobacters, necessitating the use of more tedious methods such as agar dilution or broth microdilution. Another problem is that breakpoint values for resistance have not been established nor has the effect of microaerophilic incubation on the MICs of antimicrobial agents been accurately determined. With disk sensitivity methods, high levels of errors occur with some antibiotics (Vanhoof et al., 1984). Also, there is no agreement among various authors about test conditions such as the optimal medium, the use of blood supplementation and temperature and time of incubation. The commercially available E-test (AB Biodisk, Solna, Sweden) has been described as comparable with standard

antimicrobial susceptibility tests such as agar microdilution and broth microdilution methods (Baker, 1992, Tenover et al., 1992). Therefore, the E-test can be used as a simple, rapid and accurate test to determine the MIC of *C. jejuni* isolates.

Monitoring of resistance patterns by antimicrobial susceptibility testing of *C. jejuni* is important because of the possibility of the organism acquiring resistance and different mechanisms and perhaps ecological differences in GI tract due to different feeds may play a role. The development of resistance to fluroquinolones has implications in the identification of campylobacter organisms to the species level as well (Altwegg et al., 1987). Fluroquinolone resistant *C. jejuni* isolates would be resistant to nalidixic acid also and hence they may be erroneously classified as *C. lari*.

In conclusion, the present study on drug resistance using disk diffusion and E-test did not reveal any significant difference in antimicrobial susceptibility.

3.6. References

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4. FLAGELLIN GENE TYPING OF *CAMPYLOBACTER JEJUNI* ISOLATES

4.1 Background

Lack of appropriate subtyping tools has hindered epidemiological investigations of campylobacter infections in the past. In order to address this issue, a system for tracking specific strains or serotypes of *Campylobacter* strains would be desirable for studying the epidemiology of human infections, analyzing modes of transmission and devising and assessing intervention strategies important in animal agriculture.

Several methods have been described for typing *Campylobacter* spp. including biotyping, serotyping, phage typing, plasmid typing, enzyme profile analysis, restriction digest analysis, pulsed field analysis and ribotyping. In particular, serotyping to detect heat labile (HL) antigens or O (heat stable) antigens has played an important role in understanding the epidemiology and identifying the source of campylobacter infections in the past (Patton, et al., 1991). Serotyping schemes are useful but have certain limitations. They require reagents that are expensive and time consuming to produce (Patton and Wachsmuth, 1992). As a result very few laboratories provide serotyping facilities and so this technique is not easily accessible to diagnosticians. Furthermore, reliance on the results of serotyping alone could be misleading and confusing (Salama et al., 1990). *Campylobacter* serotypes are not species specific and the influence of environmental factors on the stability of most of these phenotypic properties is another major weakness of these techniques (Yan et al., 1991). Phenotypic techniques have a high untypeable rate in veterinary isolates (Newell et al., 2000). These drawbacks restrict their application for investigating likely sources of *Campylobacter* spp. causing infections in humans.

Molecular methods such as restriction endonuclease analysis (Patton et al., 1991), analysis with genes encoding for rRNA (Kiehlbauch et al., 1991), plasmid analysis (Bradbury et al., 1983), multilocus enzyme electrophoresis analysis typing (Aeschbacher and Piffaretti, 1989) have become popular as alternatives to serological methods recently.

Restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) products of the *Campylobacter* flagellin gene (*fla A*) known as flagellin gene typing, is a useful molecular based method (Nachamkin et al., 1996) and can be used as a practical typing method for evaluating the phylogenetic and epidemiological relationships among strains. In epidemiological analysis, the stability and reproducibility of strain identification systems are critical. According to Fujimoto et al. (2000) and Peterson and Newell, (2001), the *fla A* RFLP patterns of *C. jejuni* strains are sufficiently stable for epidemiological analysis.

The purpose of this study was to confirm that the isolates recovered from the experimental birds were identical to the challenge strain or that the genotype of the isolates had undergone any changes.

4.1.1. Flagellin genes

Campylobacter flagella are needed for colonization in the intestinal tract (Lee et al., 1986). Nonflagellated variants are incapable of colonizing the intestinal tract of animals or humans (Caldwell et al., 1985 and Black et al., 1988). The motility of flagella is absolutely necessary for colonization in the GI tract and hence flagella are considered to be an important virulence factor. They undergo both phase (aflagellate, nonmotile) and antigenic variations (Caldwell et al., 1985 and Harris et al., 1987). Flagella are the major serodeterminant in

certain serogroups of the HL serotyping scheme (Harris et al., 1987). The subunit flagellins are glycosylated and flagellins are posttranslationally modified (Doig et al., 1996). The presence of glycosylated proteins in bacteria is itself highly unusual and raises the possibility of molecular mimicry to glycosylated moities on human proteins (Guerry, 1997). *Campylobacter* flagellin shows a high degree of sequence similarity to the flagellins of *Salmonella* in the amino and carboxy ends of the molecules (Logan et al., 1989), which are known to be conserved among bacteria and which have been shown to function in the transport and assembly of the monomers into flagellar filaments in enteric bacteria (Homma et al, 1987). The internal regions of *Campylobacter* flagellin contain antigenically variable sequences.

There are two adjacent tandemly oriented flagellin genes (Guerry et al., 1991) named *fla A* and *fla B* each approximately 1.7 kb in size and separated by an intergenic space region of 170 bp (Genbank accession # AF 050197 vide Fig 4.1). The open reading frames are 95% identical at the nucleotide level. Differences between *fla A* and *fla B* occur mainly in the 5' and 3' ends and in a small region in the middle of the genes. The genes are similar only within the coding regions while their upstream regions show no significant similarity. These genes are transcribed concurrently although their transcription is regulated by separate promoters. The *fla A* gene is controlled by a σ^{28} promoter while the *fla B* gene by a σ^{54} promoter. Both *fla A* and *fla B* gene products are capable of forming functional flagellar filaments. Mutations in the *fla A* gene produces nonmotile cells where as mutations in *fla B* locus produces cells with decreased motility and a truncated flagellar structure (Guerry et al., 1991). The tandem arrangement of flagellin genes suggests that the two genes originated from a duplication

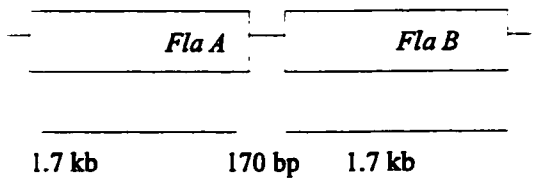
event. Perhaps *C. jejuni* retained both genes so as to have an alternate copy for recombination repair in response to a mutation event in one gene (Guerry et al., 1992). Also, a filament composed of both subunits confers greater motility than a filament of equal length composed of only the Fla A subunit and the increased motility appears to enhance the ability of the organism to colonize the intestinal tract. *Campylobacter* spp. have the ability to modulate the expression of the *fla B* gene in response to environmental signals. This suggests that there is a mechanism by which campylobacters may control their motility status, which in turn, may modulate virulence (Guerry, 1997).

The flagellin gene *flaA* in *Campylobacter* spp. appears to have significant sequence heterogeneity so that molecular analysis of the flagellin genes might serve as a good epidemiologic marker. Computer assisted restriction site analysis of the sequence data showed that although *fla A* and *fla B* are more than 93% homologous, digestion with restriction enzyme *Alu* I would give different digestion patterns (Thomas et al., 1997). Furthermore, between strains the *fla A* genes may differ by as much as 30% indicating that flagellin gene typing can discriminate between strains within a particular serotype. The restriction map of the enzyme *Dde* I (created with Clone Manager 6) is shown in Fig 4.2.

Spontaneous intragenomic flagellin recombination has been reported both for *C. coli* and *C. jejuni.* Studies of *C. coli* infections in pigs suggested that the organism was not clonal and could demonstrate considerable genetic variation during the course of an intestinal infection (van der Plas et al., 1995). Isolation of revertant motile *C. coli* strains in which intragenomic recombination resulted either in deletion or in repositioning of Km^r cassettes initially located in the *fla A* gene has been described (Alm et al., 1993). Similar motility

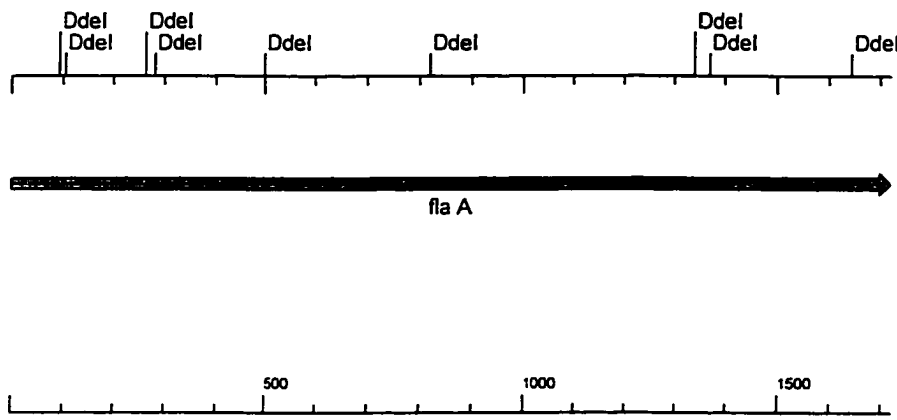
revertants from immotile mutant *C. jejuni* strains have also been reported (Wassenar et al., 1995). Nuijten et al. (2000) have reported that a nonmotile *fla A* mutant of *C. jejuni* underwent rearrangements within its flagellin locus, and regained its motility and colonization capacity, during colonization of chicken intestine. Also, intergenomic recombination (horizontal gene transfer) has been demonstrated between the flagellin loci of two mutant *C. jejuni* strains each containing different antibiotic resistance markers in their flagellin genes (Wassenar et al., 1995). Harrington et al. (1997) showed indirect evidence for intragenomic *fla* recombination in a nonmutant strain and for intergenomic recombination within natural populations of *C. jejuni* by sequencing many *fla A* and *fla B* genes. Hanninen et al. (1999) have described recombinations between *Campylobacter* strains after co-colonization of these strains in the chicken intestine. These studies provide increasing evidence that genetic recombination can occur in campylobacters and may raise doubts about the long term stability of *fla A* genes. The purpose of this study was to confirm that the isolates recovered were identical to that used for challenge of the experimental birds.

Fig 4.1. Schematic representation of Flagellin genes of *Campylobacter jejuni*.



Both *fla A* and *fla B* genes of *C. jejuni* are approximately 1.7 kb and are arranged in a tandem orientation with an intergenic space of 170 bp.

Fig 4.2. *Dde* I Restriction map of *fla A* gene of *Campylobacter jejuni*



fla A (1719 bps)

4.2. Materials and methods

4.2.1. Isolates used in the study

The *Campylobacter jejuni* isolates used in the study came from birds used in the dietary study described in Chapter 2. Out of the isolates recovered, 40 isolates were randomly selected using a computer based software program (Minitab 12 Inc, Version 12) and were then characterized and confirmed as *Campylobacter jejuni* using morphological and biochemical tests, and were subjected to molecular characterization with respect to *fla A* gene. The same isolates were also sent for biotyping and serotyping studies to National Laboratory for Enteric pathogens, Canadian Science Center for Human and Animal Pathogens, Winnipeg, Manitoba, Canada.

4.2.2. Extraction of genomic DNA

The selected *C. jejuni* isolates were grown on BA (Oxoid) plates under microaerophilic conditions providing 5% oxygen, 10% carbon dioxide, and 85% nitrogen at 42° C overnight. Genomic DNA was extracted using a commercially available kit (Promega™ Wizard Genomic DNA purification kit). The extraction process involved the following steps using the reagents supplied with the kit. A 10 μL loopful of bacterial colonies was transferred to 600 uL of lysis solution in a 1.5 mL microcentrifuge tube and was incubated at 80° C for 5 minutes in a water bath to lyse cells and then cooled to room temperature. Three microliters of RNAse solution were added to the cell lysate and tubes were inverted 5 times to mix thoroughly. The tubes were then incubated at 37° C for 1 hour in a water bath and cooled to room temperature. Two hundred μL of protein precipitation solution were added and mixed by vortexing vigorously at high speed for 20 seconds and then incubated on ice for 5 minutes.

Then it was centrifuged at 13, 000x g for 3 minutes. The supernatant containing DNA was transferred into a clean 1.5 mL microcentrifuge tube containing 600 μL of isopropanol, gently mixed by inversion and centrifuged at 13, 000 x g for 2 minutes. The supernatant was carefully decanted and the DNA pellet was carefully washed with 600 μL of 70% ethanol. Following centrifugation for 2 minutes at 13, 000x g, ethanol was decanted and the pellet was allowed to air dry for 15 minutes. One hundred microliters of DNA rehydration solution were added to the tube and rehydrated the DNA by incubating overnight at 4° C. The concentration of DNA in the extracts was determined using a spectrophotometer (Ultrospec II, 4050 UV/ visible Specrophotometer, LKB Biochrom). Microcuvettes which hold approximately 700 μL volume were used. Deionized distilled water was used as the reference blank and for making dilutions. 1 in 100 dilutions of the extracts were prepared and optical density at a wave length of 260 nm initially and at 280 nm subsequently was read using the spectrophotometer.

4.2.3. Amplification of *fla A* gene using Polymerase chain reaction (PCR)

A mastermix was prepared by adding components in the following order. For 50 μL reaction sterile double distilled water, 37 μL; 10 X PCR buffer, 5 μL; dNTP mix (0.2 mM each), 1 μL; forward and reverse primers (2.5 pM each), 1 μL each; Taq DNA polymerase (1U) (polymerase and reagents from Biofermentas, Ontario, Canada), 1 μL and 25mM magnesium chloride, 3 μL. To this 1 μL of DNA (~ 40 μg/ μL) template was added. The primer sequences were consensus sequences based on published primer sequences (Nachamkin et al., 1997) and BLAST search (Altschul et al., 1997) using *C. jejuni* strain D 1118 as the querry sequence (Genbank, Pubmed Accession # AF 050197). The forward

primer consisted of (5'- 3') ATG GGA TTT CGT ATT AAC AC(A/C) AAT G and reverse primer of (5' - 3') CTA CTG TAG (C/T) AA TCT TAA AAC ATT (Bio/ Can Scientific, Mississauga, Ontario, Canada). As a negative control along with the above components, 1 μL sterile double distilled water was used instead of template DNA. The PCR was performed using a thermal cycler (Hybaid PCR Sprint, UK) under the following conditions- stage 1, step 1, denaturation at 95° C for 5 min; stage 2, step 1, 95° C for 45 sec; step 2, annealing at 55° C 45 sec; step 3, 72° C 40 sec for 25- 30 cycles and stage 3, step 1, elongation for 3 min. After completion of the amplification the presence of PCR products were detected by agarose gel electrophoresis as described below.

4.2.4. Electrophoresis of PCR products

The gel for electrophoresis was prepared by dissolving 1g of agarose (GIBCO, BRL, MD, USA) in 100 mL of 1X TAE buffer (vide appendix C) in a microwave oven. Then the gel was electrophoresed (BioRad Power Pac 300) for 45 minutes at 100 V in 1X TAE buffer containing 6 μL of ethidium bromide (10mg/ mL) and the gel was visualized and photographed using a computer based gel imaging system (Syngene Gene Snap Gel documentation system, UK).

4.2.5. RFLP analysis

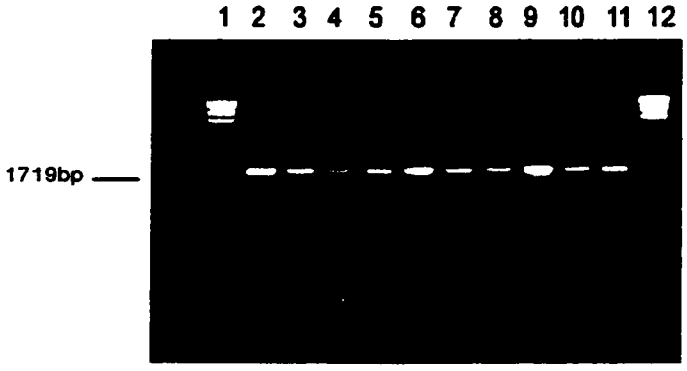
To 40 μL of PCR product, 5 units of *Dde* I enzyme (Promega) and 2.5 μL of 10X BSA were added. The reaction was incubated for 2 hours in a 37° C incubator. The digested samples were loaded onto 4% Nusieve GTG agarose gel (BMA, Maine, USA). The gel was electrophoresed for 4 hours under 85 V (BioRad Power Pac 300) at room temperature containing 6 μL of ethidium bromide (10mg/ mL) in 0.5% TBE buffer (vide appendix F).

After electrophoreses gels were destained for 1 hour in 0.5 % TBE buffer. The gel was visualized and photographed using a computer based gel imaging system (Syngene Gene Snap Gel documentation system, UK).

4.3. Results

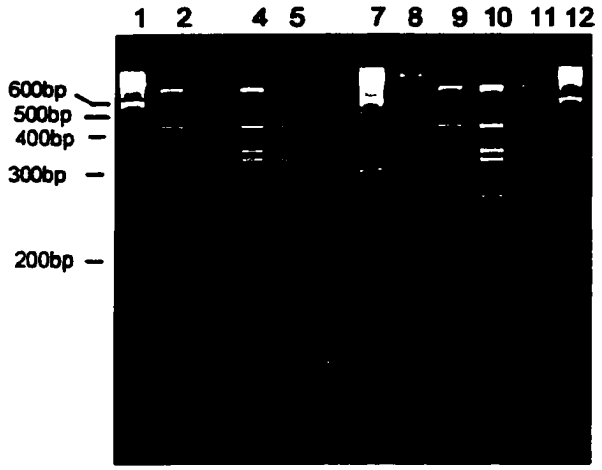
Of the 40 isolates subjected to analysis, all of them could be amplified using DNA extracted using the Promega kit. Amplicons of size 1.7 kb were obtained for all isolates and representatives of which are given in Figs .4.3. The RFLP patterns of the PCR products of isolates digested with the restriction enzyme *Dde I* belonging to the various feed groups are presented in Figs. 4.4 and 4.5. The fragments were lying between 100 bp and 800 bp bands of the 100 bp molecular weight marker. These figures presented two distinct *fla A* patterns. The strain used for infecting birds (lane 5, Fig. 4.4) and most of the isolates belonged to a single *fla A* type while several of the isolates showed little variations. The comparison of results between the *fla A* gene typing and serotyping are given in Tables 4.1.1 and 4.1.2. Only two isolates (5%), designated 8/ 1 (lane 8 fig. 4.4) and 8/ 2 (lane 5 fig. 4.6) showed variation in band patterns in comparison to others.

Fig 4.3. Amplification of 1719 bp *fla A* gene of *Campylobacter jejuni* isolates.



Legends: Lanes 1 and 12. λ Hind III marker (Promega). Isolate # s from Lane # 2. 9/2, 3. 5/2, 4. 6/4, 5. 4/1, 6. 1/1, 7. 6/5, 8. 5/4, 9. 12/1, 10. 5/3, 11. 10/1. The size of the PCR product is indicated on the left side.

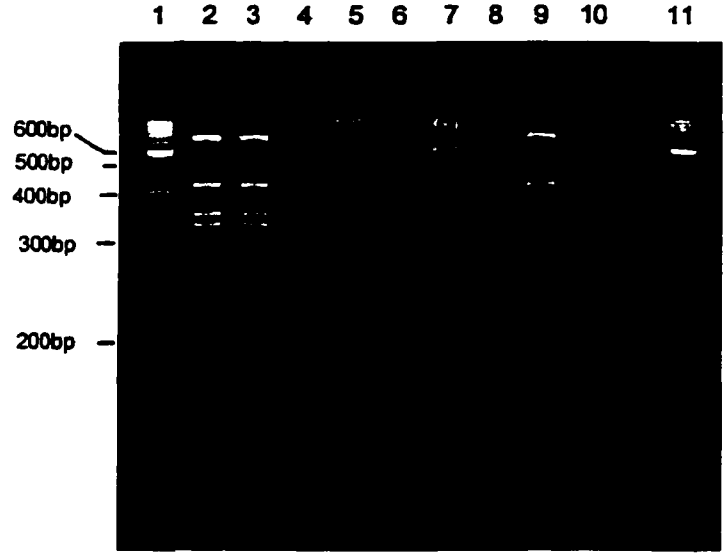
Fig. 4.4. *fla A* gene RFLP fingerprints of *Campylobacter jejuni* isolates.



Legends: Lanes 1, 7 and 12 contain the 100 bp molecular weight marker (ladder). Lane # 2. 5/ 1, 4. 7/2, 5. 13/1 (strain inoculated). 8. 8/1, 9. 10/ 3, 10. 1/3, 11. 4/2.

Lane 8: isolate showing variation.

Fig. 4.5. *fla A* gene RFLP fingerprints of *Campylobacter jejuni* isolates.



Legends: Lanes 1, 7 and 11 contain 100 bp ladder. Isolate #s from Lane #s 2. 9/ 2, 3. 1/ 3, 4. 6/ 5, 5. 8/ 2, 6. 5/ 4, 8. 5/ 3, 9. 6/ 2, 10. 3/ 2. Lane #5, isolate showing variation.

Table 4.1.1. Comparison of results of flagellin gene typing and serotyping

AVC Isolate #	Source	RFLP pattern	Biotype	Serotype	
				Penner (O)	Lior (HL)
12/3	Plant	Pattern 1	IV	O: 21, O: 29.	UT
11/1	Mixed	Pattern 1	IV	O: 21, O: 29	UT
4/3	Animal	Pattern 1	IV	O: 21, O: 29	UT
10/ 2	Animal	Pattern 1	IV	O: 21, O: 29	40
5/ 1	Mixed	Pattern 1	IV	O: 21, O: 29	UT
12/2	Plant	Pattern 1	IV	O: 21, O: 29	40
7/2	Mixed	Pattern 1	IV	O: 21, O: 29	UT
13/1*		Pattern 1	IV	O: 21, O: 29	UT
12/1	Plant	Pattern 1	IV	O: 21, O: 29	40
8/1	Plant	Pattern 2	II	O: 2	4
10/ 3	Animal	Pattern 1	IV	O: 21, O: 29	40
1/3	Mixed	Pattern 1	IV	O: 21, O: 29	UT
4/2	Animal	Pattern 1	IV	O: 21, O: 29	UT
2/2	Plant	Pattern 1	IV	O: 21, O: 29	UT
4/1	Animal	Pattern 1	IV	O: 21, O: 29	UT

Legends: *strain used for challenge; UT : untypeable.

Table 4.1.2. Comparison of results of flagellin gene typing and serotyping (contd.).

AVC Isolate #	Source	RFLP pattern	Biotype	Serotype	
				HS (Penner)	HL (Lior)
1/2	Mixed	Pattern 1	IV	O: 21, O: 29	UT
2/ 3	Plant	Pattern 1	IV	O: 21, O: 29	UT
6/ 1	Plant	Pattern 1	IV	O: 21, O: 29	UT
9/ 1	Animal	Pattern 1	IV	O: 21, O: 29	UT
3/ 4	Animal	Pattern 1	IV	O: 21, O: 29	UT
7/ 3	Mixed	Pattern 1	IV	O: 21, O: 29	UT
11/ 2	Mixed	Pattern 1	IV	O: 21, O: 29	UT
5/ 2	Mixed	Pattern 1	IV	O: 21, O: 29	UT
6/ 6	Plant	Pattern 1	IV	O: 21, O: 29	UT
10/ 1	Animal	Pattern 1	IV	O: 21, O: 29	40
9/ 2	Animal	Pattern 1	IV	O: 21, O: 29	40
1/ 3	Animal	Pattern 1	IV	O: 21, O: 29	UT
6/ 5	Plant	Pattern 1	IV	O: 21, O: 29	UT
8/2	Plant	Pattern 2	II	O:2	4
5/ 4	Mixed	Pattern 1	IV	O: 21, O: 29	UT
5/ 3	Mixed	Pattern 1	IV	O: 21, O: 29	UT
6/ 2	Plant	Pattern 1	IV	O: 21, O: 29	UT
3/ 2	Animal	Pattern 1	IV	O: 21, O: 29	UT

4.4. Discussion

In this study one isolate of *Campylobacter jejuni* (biotyped and serotyped as biotype IV, serotypes HS O:21, O: 29, HL untypeable) was used to challenge birds belonging to different treatment groups (vide chapter 2) and subsequently organisms were recovered from the birds once every three days from the day following infection till the end of study period. All birds were quarantined observing all biosecurity protocols and hygienic measures to protect them from environmental exposure in order to prevent colonization of any other type of *C. jejuni* strains.

The purpose of this study with flagellin gene typing was to determine whether all isolates recovered from the birds were same as the one that was as inoculum. Of the isolates recovered from birds, 40 were randomly selected for characterization by various methods including a molecular method and serotyping. It was found that in *fla A* gene typing 95% of the isolates recovered had the same restriction enzyme profile as the strain used for inoculation. However, 5% of isolates showed variation in band pattern. This showed that there is potential for some variability, perhaps due to genomic rearrangements of those isolates occurring in the intestinal tract of birds. However, it is worth exploring the potential for mutagenicity of diet formulations. Also, that RFLP typing was able to depict the level of genotypic variation in *Campylobacter jejuni* isolates and has potential for clearly discriminating them. The results of the RFLP analysis agreed with that of serotyping with HS (O) scheme and the biotyping scheme.

The discrimination potential of flagellin gene typing is as good as that of serotyping schemes and would suffice for most practical purposes. However, some workers have

indicated that recombination within or between flagellin loci occur in *Campylobacter* strains suggesting the instability of the genome (Harrington, 1997, Hanninen, 2000 and Nuijten et al., 2000). Therefore, it would be desirable to use the fingerprinting of other conserved and universally distributed genes eg., 23s rRNA gene, along with *fla A* gene when better accuracy of results are required in epidemiological typing.

4.5. References

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5. GENERAL DISCUSSION

Poultry and poultry meat have often been implicated as major vectors in the transmission of the most common human foodborne bacterial enteropathogen, *Campylobacter jejuni,* which has emerged recently as a significant public health concern throughout the world. These organisms colonize the intestinal tracts of birds and are usually transmitted onto their carcasses during processing. In order to reduce the level of contamination of processed meat with *Campylobacter*, chickens with a reduced intestinal load of this pathogen should be made available for processing because attempts directed at improving microbiological quality of poultry meat at the processing end are not very successful practically. Therefore, control of these organisms at the farm level during the growth of the broiler chicken is crucial. If an effective intervention strategy could be worked out, it would result in a cleaner product for the consumer, thereby reducing the incidence of campylobacteriosis. In order to commercially raise chickens with a reduced load of campylobacters, various factors that influence their colonization should be well understood. The main barrier in the progress of intervention strategies is the lack of sufficient knowledge of the basic microbiology of intestinal colonization of these organisms and factors that influence the composition and interactions of microflora.

Preventing birds from being colonized with *C. jejuni* is difficult because of the commensal association they have with other chickens. The commensal relationship also hinders immunological intervention strategies. *Campylobacter jejuni* was implicated as the cause of a condition called avian vibrionic hepatitis, a disease that was frequently encountered in poultry flocks in America and Europe during the 1950's and 1960's. However,

this disease is not generally encountered in commercial flocks at present. Perhaps, the implicated pathogen responsible for the early cases was different or host adaptation of *C. jejuni* had occurred.

The food animal industry is very much dependent on the use of chemicals and drugs, particularly antibiotics, for the welfare of animals as well as the economy of the industry. The indiscriminate use of antibiotics, however, in animal production may lead to serious human health hazards because of antibiotic residues and emergence of antibiotic resistant spp. of microorganisms. The level of antibiotics used as feed additives in growing birds may not be effective in preventing them from becoming colonized with *Campylobacter* spp. (Engvall et al., 1986). Another problem is that the use of antibiotics would affect the indigenous microflora thereby favoring the colonization of pathogenic organisms. The USFDA recently announced its plan to ban the use of two antibiotics in poultry in the fluroquinolone family. The reason stated for the justification of its action is the potential of emergence of fluoroquinolone resistant *Campylobacter* organisms (Cimons, 2001). It is most likely that the use of other antibiotics in poultry and other food animals also could be banned in the near future because of emerging human health concerns. So on a public health point of view it becomes necessary to develop nonantibiotic alternatives in order to reduce the use of antibiotics in animal agriculture without compromising the health and productivity of animals.

Adhering to good sanitary protocols and hygienic measures are effective in preventing the colonization of *C. jejuni* in a flock. Nonetheless, these tasks are difficult to achieve in the existing farming conditions. However, the use of sanitary protocols such as clean clothes,

footwear disinfection before entry into the poultry house, proper cleaning and disinfection of poultry houses and utensils, and design of buildings to keep away flies, rodents and other feral animals coming in contact with birds, restricting access to poultry houses, use of fresh litter for every batch, etc, may help in reducing the organism getting access to farmers' facilities. A main route of *C. jejuni* infection is drinking water. The addition of disinfectants such as chlorine may help to reduce the transmission of organisms through the drinking water. The practice of "all in all out" operations should be advocated because when some birds are depopulated at intervals, the chances for persistence of infection in remaining birds would be very high. In Scandinavian countries, where stricter biosecurity protocols are observed, the rate of flock infection is reported to be substantially lower (Kapperud et al., 1993).

The factors that affect the colonization of microorganisms include a triad comprising the organism, the host and the environment. Not only the various aspects of each component of organism- host- environmental interrelationship are essential but also their fine orchestration in accordance with the environmental cues is necessary for proper colonization. Of the various environmental factors studied so far, the effect of diet on colonization of *C. jejuni* has not been addressed properly.

The research reported in this work has examined how three different diet formulations influenced the colonization of *Campylobacter jejuni* organisms in the intestinal tract of broiler chickens. The strain used for challenge of birds and isolates recovered from the birds in the experimental groups were characterized using standard methods, serotyping, the antimicrobial sensitivity profile of commonly used antimicrobial agents and a genotyping method.

In Chapter 1, a detailed review of the literature examined general aspects of the

microbiology of *C. jejuni*, followed by a discussion on the various factors that influence, and how these factors interact in, the colonization of microorganisms with particular attention to the colonization of *C. jejuni* in the intestinal tract of chickens, and finally the previous studies on interaction of various dietary factors in the colonization of microorganisms.

A strategy that has been tried in preventing colonization of pathogens in the GI tract of birds is the manipulation of indigenous microflora. Several approaches have been tried with varying degrees of success in this regard. The major approaches so far tried, have been directed at the use of prebiotics, probiotics and synbiotics in poultry. All these strategies, invariably manipulate the gastrointestinal microflora so that growth of some beneficial organisms is favored to suppress the colonization of pathogens. But some of the strategies reported to be helpful in reducing the colonization of *Salmonella* spp. have been found not that useful in the case of *Campylobacter* spp. This is because there are certain fundamental differences in the epidemiology of these organisms. Usually, birds become colonized with *Salmonella* during the second or third week of growth and there occurs a gradual decline in colonization toward the marketing age. In the case of campylobacters, although colonization occurs around the second or third week, once the organism appears in a flock it rapidly spreads, and practically all birds become colonized within a few days and remain heavily colonized throughout the growout period (Shreeve et al., 2000). In the present study it was also found that the intestinal tract of birds were heavily colonized with *C. jejuni* throughout the study period. *Salmonella* colonizes the epithelium of the lower intestinal tract mainly the cecum whereas *Campylobacter* are found freely swimming in the mucin layer. Hence, strategies that target organisms found in the epithelium, such as receptor antagonism may not

be very helpful in the case of *Campylobacter*. Therefore, it is desirable to concentrate further attention to identifying suitable mucous and crypt dwelling organisms whose use as part of CE flora may help to competitively exclude *Campylobacter*.

The indigenous microflora in the GI tract of birds play an important role in the metabolism of gut lumen and colonization of pathogens (Savage, 1986).The microbial dynamics of the GI tract is influenced by many dietary factors as well. Therefore, it is of interest to develop nutritional alternatives to manipulate the GI tract flora in order to cause a reduction in the colonization of *C. jejuni*. The literature presents a number of reports of dietary components modifying immune function. Feeds may contain a number of immunomodulators including β-glucans and arabino (rhamno) galactans of plant, fungal, yeast or microbial origin, saponins and phenolic derivatives. β- glucans are taken up by M cells in the gut which stimulate macrophages as well as complementary system. Thus, selected feed ingredients as well as herbal extracts may enhance or modulate immune responses (Meijer, 2001). Nutrients may impact microbial ecology by several mechanisms. Most of these mechanisms involve the immune system, while a few influence the physicochemical aspects that modify population of microorganisms in the GI tract. Although a lot of information is available on the impact of nutrients on immune system, literature on their effects on microbial colonization is scanty. This area offers potential for specially designed feeds which may modify the innate system and increase disease resistance. For optimal growth, production and disease resistance, the animal diets should be well balanced and designed to meet the requirements of disease resistance and resilience.

Diet appears to influence, along with other factors, resistance to infection because

specific nutrients are important in endocrine regulation, immune and somatic cell function, antibody production and tissue integrity. Especially, micronutrient and antioxidant interactions may increase disease resistance through regulating cellular and molecular processes, including membrane integrity and flux, superoxide formation and leukocyte function (Tengerdy et al., 1983). The response of an animal to an infection depends on its immune status while the immune status is dependent on its nutritional status. Since the immune system is not a significant consumer of nutritional substrates, the nutrients such as proteins, vitamins and minerals along with energy may contribute more to the resilience of the system than to disease resistance. Amino acids along with energy, and other micronutrients are needed in excess of normal requirements to meet the additional requirements of increased cell replication, acute phase protein synthesis, and vigorous immune response including synthesis of immunoglobulins, cytokines and eicosanoids during an infectious challenge.

Over and above the dietary factors strengthening the immune function and contributing to disease resistance, compositional factors of diet may also influence the growth or colonization of microorganisms in the intestinal tract. These factors include pH, water activity, OR potential, nutrient content and presence of certain inhibitory substances. The interaction between diet and microorganisms may be beneficial to the host some times and can be harmful at times. This gives rise to a few questions. Why is this interaction beneficial some times and harmful at other times?. What are the factors that govern these interactions and why do some substrates support the growth of certain organisms more readily than others? To address these issues we have to understand the interaction of diet with

microorganisms in the gut. Diet is the substrate, and hence the characteristics of its components become important. The diet or substrate influences very much in determining what organism is to be favored or disfavored. Also, the types and population of microorganisms, the species of the host and the environmental conditions that prevail in the GI tract are significant. At any given time, each diet has its characteristic microbial profile which changes as environmental conditions change. Accumulation of metabolic products may limit growth of certain species. Organisms which cannot compete in a particular environment are gradually eliminated. Gastrointestinal microflora are acquired during early stages of life, the composition of which is dependent on feeding. By understanding the characteristics of dietary components or the substrates and the environmental conditions we may be able to make predictions about the microbial populations that are likely to be supported or favored for growth. This knowledge can be effectively exploited for the nutritional manipulation of the microbial flora in the GI tract of birds.

In the work presented in Chapter 2, the main objective was to determine how diet formulations containing proteins from different sources influenced the colonization of *Campylobacter jejuni* in the intestinal tract of birds. This was done by feeding three different diet combinations similar in energy and protein contents but differing in sources of proteins to broiler birds from day one to marketing age and by determining their effect on the colonization at slaughter age by enumerating the intestinal load (cfu/ g) in organs found commonly colonized.

The isolation of *Campylobacter jejuni* from chickens, standardization of inoculum for infecting birds, different aspects of colonization and recovery of *C. jejuni* from birds,

identification of isolates and serotyping have been discussed.

Newly hatched chickens were found free of *C. jejuni* in the present study which agreed with previous reports (Clark and Bueschkens, 1985; Engvall et al., 1986; Pokamunski et al., 1986). The strain of *C. jejuni* used in the study was found to readily colonize the intestinal tract of birds without causing any adverse effects on the birds. A single dose oral administration was sufficient to establish and maintain the organism in the GI tract of birds which resulted in shedding of organisms till the end of marketing age. Sampling with calcium alginate swabs was found to be an effective method for the recovery of *C. jejuni* from the cloaca of birds. The cloacal shedding of organism indirectly reflected intestinal colonization. The intensity of growth of organisms in the plates cultured from cloacal swabs may be a close approximation of the degree of colonization and hence may be used as a noninvasive test to determine intestinal colonization. A rising and falling shedding pattern was observed in the main study, which agreed with a previous report (Achen et al., 1998). However, a continuous pattern of shedding was noticed in the pilot study. The difference noticed in the pilot study may perhaps be due to small sample size of birds used. The reason for cyclical shedding is not clearly understood but may correspond to the cyclical emptying of cecal contents.

It was obvious in the analysis of data on cfu/ g of intestinal organs quantitatively cultured, that there was an organ dependent, significant interaction between the feeds in the study group and colonization of *C. jejuni*. The colonization of *C. jejuni* in the ceca of birds in the plant protein based feed group was found to be significantly lower than the other groups. But the difference in the colonization of organisms in jejuni or crops were

insignificant. However, the noticed reduction in colonization in the ceca of birds fed with plant protein based feed appears to be of practical significance because the main cause for carcass contamination is through the onward transmission of organisms from the rupture of ceca occurring during the mechanized evisceration process.

The mechanisms involved in the observed reduction of colonization in the birds fed with plant based feed were not examined as part of this work. However, in order to explain the reasons behind the noticed difference the following possible underlying mechanisms are discussed. In the case of birds, the main substrate that affects the microflora in the GI tract is the carbohydrates and this effect is dependent upon the type of carbohydrate. The review of literature suggests that the presence of water soluble carbohydrates increases microbial activity. The water soluble NSPs present in barley, rye or wheat have been found to increase the activity of microorganisms in the gut (Choct and Annison, 1992). Fermentation of nondigestible dietary substrates is a major metabolic function of microflora which results in the formation of products such as gases, hydrogen, carbon dioxide, short chain fatty acids (SCFA), lactic acid, ethanol, branched chain fatty acids (BCFA), ammonia, amines, phenols and indoles (Roberfroid, et al., 1995). Gases are the main products of microbial metabolism. The gases and acid produced during fermentation accumulate to a certain capacity level which causes the faster emptying of the cecal contents. The comparative enlargement of ceca of birds in the plant protein group observed in this study is indicative of fermentation process taking place in the organ. Fermentation activity in the ceca is suggestive of presence of substrates which favor microbial fermentation. Fermentation of substrates by some organisms may result in the creation of a restrictive physiologic environment which is inhibitory to other

organisms. The SCFA and BCFA formed as end products of microbial fermentation of carbohydrates and proteins, respectively, could be inhibitory to some organisms. As a result of production of carboxylic acids, the pH of luminal contents becomes more acidic. The change in the flora may lower pH and a decrease in putrefactive products resulting in the accumulation of acetate and in the increase of volatile fatty acids (Corrier et al., 1990; Waldroup et al., 1993). The reduced cecal pH and OR potential enhance the antibacterial activity of VFA. Control of the anaerobic metabolism of peptides and proteins by microflora is less understood than that of carbohydrates and further research is needed in this regard.

Some feed compositions have capacities to induce certain lectins designated as Feed Induced Lectins (FILs) as discussed by Gorranson et al. (1993). These authors observed that FILs could reduce diarrheal diseases in pigs by inhibiting small intestinal hypersecretion of water and electrolytes. Lectin induction might probably be a mechanism that may influence the intestinal colonization of microorganisms.

In this study birds belonging to groups fed with a feed formulation containing plant protein sources were found to harbor the lowest number of *C. jejuni* in comparison to groups fed with animal protein based feed or mixed protein feed. The presence of animal grease in the plant protein based feed may have been protective for *C. jejuni* and probably have affected the results to some degree. The effect would have probably been more if the diet was designed exclusively of plant ingredients. However, the results of previous studies on dietary interactions and microflora had often been reported inconsistent and unpredictable due to the following reasons. The level of nutrients and their bioavailabilities may vary between diets and even between different batches of the same feedstuff. In addition, the effects of various

nutrients are more or less overlapping and are nonexclusive. Therefore, it would be desirable to check the consistency of results of this experiment in repeated trials.

In future studies, it would be desirable to under take the mechanistic aspects by measuring the changes that take place in the ceca of birds consequent to fermentation. The changes occurring in pH, OR potential, levels of VFA produced, induction of lectins, differences in iron content may be examined.

A suitable diet formulation may have an added effect when strategies such as CE or synbiotics are employed. A combined use of CE strategy with prebiotics on a diet designed exclusively of plant based components may contribute a great deal in reducing the colonization of *C. jejuni* in the GI tract of birds. Identification of mucous and crypt dwelling organisms which could competitively exclude *C. jejuni* may strengthen the CE strategy. Improved biosecurity protocols along with good water supply may effectively reduce the colonization of organisms in the GI tract of birds and help to deliver birds with much reduced intestinal load of *C. jejuni* to the processing plants. Research on development of chickens resistant to the colonization of *C. jejuni* and immunological strategies should also be strengthened. A total elimination of *C. jejuni* appears not feasible at this moment and it is doubtful that this can ever be achieved. It would be beneficial to the consumers if some precautions in the processing procedures could be under taken. Prevention of processing plant cross contamination is an important area to be focused to reduce the *C. jejuni* load on carcasses sold to the consumers. Methods for evisceration should be improved to prevent the rupture of viscera during processing. The cages and vehicles used for transport of birds should be decontaminated after each use. Spray washing of carcasses should be done prior to and

after scalding and chilling. Although *C. jejuni* do not survive for long periods outside the host, a major factor contributing to their survival on chicken carcasses is the presence of higher amounts of available water and hence measures for reducing the available water on chicken carcasses would be beneficial in reducing the incidence of campylobacteriosis.

On the consumer side, the proper cooking of poultry meat would prevent the incidence of campylobacteriosis. Cross contamination of *C. jejuni* from chicken meat to foods eaten uncooked should be avoided. For this the hygienic aspects in the food handling procedures currently practiced should be improved. By giving better attention to all the above listed measures the problem of campylobacter infections in humans can be reduced. The experimental approaches used in this work could serve as a model for further research relating to dietary manipulation of *C. jejuni* colonizing the intestinal tract of broiler birds.

In Chapter 3, the determination of antimicrobial sensitivity profile and minimum inhibitory concentration (MIC) of isolates was described. In this study, antibiotic sensitivity of isolates were determined using disk diffusion method and MIC with E-test. As *C. jejuni* has emerged as the most important cause of bacterial enteritis in humans, the determination of antimicrobial susceptibility and MIC is important for identification of antimicrobial agents to which the organisms have become resistant and strains with special resistance patterns. Antimicrobial resistance among campylobacters is on the rise and hence it is important to track down the emergence of resistance by periodical susceptibility testing, for surveillance and epidemiological purposes. All of the isolates in this study including the challenged strain were found to be resistant to tetracycline. The data analysis showed that there was no significant difference between isolates belonging to the three feed groups

subjected to the study and isolates from first and last days of recovery in both antimicrobial sensitivity patterns and MIC values. This showed that difference in feed composition was not a factor with regard to the antimicrobial sensitivity profiles of *Campylobacter jejuni* isolates in this study.

In Chapter 4, the work on the molecular characterization of isolates has been described. The method used was flagellin gene typing in which the *fla A* gene of *C. jejuni* was amplified using PCR and then subjected to RFLP fingerprinting. The pattern observed showed that 95% of isolates belonged to one pattern while 5% exhibited a little variation. The results suggested that there is potential for variability probably due to genetic rearrangements that may occur among *C. jejuni* in the intestinal tract of birds. The results of biotyping and serotyping with HS antigens also agreed with this observation. 95% of isolates belonged to biotype IV, serotype HS O: 21, O: 29 while 5% to biotype II serotype HS O: 2. However, in HL typing most of the isolates were reported untypeable while a couple of them were HL 4 type and a few were HL 40 type. The reason may be that the HL system apparently does not cover all existing serotypes, particularly animal isolates. The results demonstrate that both serotyping of isolates with HS antigens and RFLP analysis of *fla A* gene are comparable and have adequate potential in discriminating between *C. jejuni* isolates.

In conclusion, this study demonstrated that a plant protein based feed compared to animal protein based feed caused significant reduction in cecal colonization of *Campylobacter jejuni* in growing broiler chickens. The antimicrobial susceptibility patterns of *C. jejuni* isolated from experimental birds were not influenced by a difference in feed composition. The drug susceptibility profiles in comparison with serotyping and *fla A* gene fingerprinting

helped to determine that the challenged strain and the colonizing strains were identical with no significant variation.

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Appendix A

Composition of rations and NRC recommended protein requirements

Composition of Chick starter ration (Purina Start & Grow* 6039)

Crude protein	20.0%
Crude fat	3.5%
Crude fiber	5.0%
Sodium	0.18%
Calcium	1.0%
Phosphorus	0.85%
Vitamin A	12,000 IU/ Kg
Vitamin B_2	2000 IU/ Kg
Vitamin D_3	20 IU/ Kg

Protein and amino acid requirements of broiler chickens (NRC, 1994)

Nutrient	0-3 weeks	3-6 weeks
crude protein	23.0	20.0
arginine	1.25	1.10
glycine + serine	1.25	1.14
histidine	0.35	0.32
isoleucine	0.8	0.73
leucine	1.2	1.09
lysine	1.1	1.0
methionine	0.5	0.38
methionine + cystine	0.9	0.72
phenyl alanine	0.72	0.65
phenyl alanine + tyrosine	1.34	1.22
proline	0.65	0.55
threonine	0.8	0.74
tryptophan	0.2	0.18
valine	0.90	0.82

Appendix B
Composition of media used

Campylobacter blood free selective medium(CBF, Oxoid)

	g/ L
Nutrient broth # 2	25.00
Bacteriological charcoal	4.00
Casein hydrolysate	3.00
Sodium desoxycholate	1.00
Ferrous sulphate	0.25
Sodium pyruvate	0.25
Agar	12.00

Suspend 22.75g in CBF selective agar base to 500 mL of distilled water and bring to the boil to dissolve. Sterilize by autoclaving at 121^0 C for 15 min. Cool to 50^0 C. Aseptically add 1 vial of CCDA selective supplement (SR 155) reconstituted with 2 mL of sterile water. Mix well and pour in to sterile petri dishes.

Blood Agar (BA, Columbia Agar base, Oxoid)

	g/ L
Special peptone	23.0
Starch	1.0
Sodium chloride	5.0
Agar	10.0
Defib. sheep blood (Oxoid)	50.0

Suspend 39 g in 1 L of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121^0 C for 15 min. Cool to 50^0 C and add 5% sterile defib. blood.

Brain Heart Infusion (BHI, Oxoid)

	g/ L
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Protease peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5

Add 37 g to 1 L of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121^0 C for 15 min.

Peptone water dehydrated (Bacto- Difco)

Peptone	10.0
Sodium chloride	5.0

To rehydrate, dissolve 15 g in 1 L of distilled water. Dispense as desired. Sterilize in autoclave for 15 min at 15 lbs pressure at 121^0 C.

Enrichment broth (EB)

Doyle and Roman (1982).
Appl Environ Microbiol. 43 (6): 1343- 1353.

Brucella broth (Gibco)	
Lysed horse blood	7.0%
Sodium succinate	0.3%
Cysteine hydrochloride	0.01%
Vancomycin	15µg/ mL
Trimethoprim	5.15µg/ mL
Polymyxin B	20.0 IU/ mL
Cycloheximide	15.0µg/ mL

Rappaport- Vassiliadis (RV) Enrichment broth (Oxoid)

	g/ L
Soy peptone	5.00
Sodium chloride	8.00
Potassium dihydrogen phosphate	1.60
Magnesium chloride (anhydrous)	40.0
Malachite green oxalate	0.04

weigh 30 g (equivalent weight of dehydrated medium/ L) and add to 1 L of distilled water. Heat gently until completely dissolved. Dispense 10 mL volume into screwcapped bottles or tubes and sterilize by autoclaving at 115^0 C for 15 min.

Modified semi solid Rappaport- Vassiliadis (MSRV, Oxoid)

	g/ L
Tryptose	4.59
Casein hydrolysate	4.59
Sodium chloride	7.34
Potassium dihydrogen phosphate	1.47
Magnesium chloride (anhydrous)	10.93
Malachite green oxalate	0.037
Agar	2.70

Suspend 15.8 g in 500 mL of distilled water Bring to boil by frequent agitation. Do not autoclave. Cool to 50^0 C and aseptically add the contents of 1 vial of MSRV selective supplements (SR 161) reconstituted with 2 mL of sterile distilled water. Mix well and pour into sterile petri dishes. Air dry for at least 1 hour.

Mueller- Hinton Agar (MHA- Oxoid)

	g/L
Beef dehydrated infusion	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0

Suspend 38 g in 1 L of distilled water. Bring to the boil to dissolve the media completely. Sterilize by autoclaving at 121^0 C for 15 min.

Appendix C

Necropsy results

bird #	feed group	necropsy results
C 1/6	plant	ascites, hepatic necrosis
C 2/5	mixed	hepatic lipidosis, ascites
C 2/6	mixed	espondilolisthesis
C 4/7	mixed	hepatic lipidosis
T 1/2	mixed	hepatic lipidosis
T 2/2	plant	hepatic lipidosis
T 2/1	plant	ascites
T 2/3	plant	hepatic lipidosis, epicarditis
T 3/4	animal	*
T 4/6	animal	hepatic lipidosis, ascites
T 5/7	mixed	hepatic lipidosis, ascites
T 6/6	plant	fibrinous peritonitis

C- uninfected birds, T- infected birds, * no diagnosis reported.

Appendix D
Results of serotyping and biotyping

Biotypes and serotypes of *C. jejuni* isolates recovered from birds fed different diets.

Lab #	NLEP #	Source (Feed group)	NLEP results
AVC 1/ 1	01- 4595	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 1/ 2	01- 4596	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 1/ 3	01- 4597	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 2/ 1	01- 4598	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 2/ 2	01- 4599	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 2/ 3	01- 4600	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 3/ 1	01- 4601	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 3/ 2	01- 4602	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 3/ 3	01- 4603	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 3/ 4	01- 4604	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29

Biotypes and serotypes of *C. jejuni* isolates recovered from birds fed different diets.

Lab #	NLEP #	Source (feed group)	NLEP results
AVC 4/ 1	01- 4605	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 4/ 2	01- 4606	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 4/ 3	01- 4607	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 5/ 1	01- 4608	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 5/ 2	01- 4609	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 5/ 3	01- 4610	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 5/ 4	01- 4611	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 5/ 5	01- 4612	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29

Biotypes and serotypes of *C. jejuni* isolates recovered from birds fed different diets.

Lab #	NLEP #	Source (feed group)	NLEP results
AVC 6/ 1	01- 4613	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 6/ 2	01- 4614	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 6/ 3	01- 4615	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 6/ 4	01- 4616	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL 40 HS O: 21, O: 29
AVC 6/ 5	01- 4617	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 6/ 6	01- 4618	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 7/ 1	01- 4619	Mixed	No Campylobacter recovered
AVC 7/ 2	01- 4620	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29

Biotypes and serotypes of *C. jejuni* isolates recovered from birds fed different diets.

Lab #	NLEP #	Source (feed group)	NLEP results
AVC 7/ 3	01- 4621	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 8/ 1	01- 4622	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype II HL 4 HS O: 2
AVC 8/ 2	01- 4623	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype II HL 4 HS O: 2
AVC 9/ 1	01- 4624	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL 40 HS O: 21, O: 29
AVC 9/ 2	01- 4625	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL 40 HS O: 21, O: 29
AVC 10/ 1	01- 4626	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL 40 HS O: 21, O: 29
AVC 10/ 2	01- 4627	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL 40 HS O: 21, O: 29

Biotypes and serotypes of *C. jejuni* isolates recovered from birds fed different diets.

Lab #	NLEP #	Source (feed group)	NLEP results
AVC 10/3	01- 4628	Animal	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL 40 HS O: 21, O: 29
AVC 11/ 1	01- 4629	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 11/ 2	01- 4630	Mixed	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 12/ 1	01- 4631	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL 40 HS O: 21, O: 29
AVC 12/ 2	01- 4632	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL 40 HS O: 21, O: 29
AVC 12/ 3	01- 4633	Plant	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O: 21, O: 29
AVC 13/ 1	01- 4634	Strain inoculated	*Campylobacter jejuni* ssp. *jejuni* Biotype IV HL untypeable HS O:21, O: 29

Appendix E

Calculation of soluble NSP content and iron content of diet formulations used

Soluble NSP content

ingredient	dry matter %	levels in the diet (%)		sNSP % of DM	Amounts calculated (%)	
		animal	plant		animal	plant
corn	89	57.8	47.3	0.7	0.36	0.29
wheat	87	20.6	12.3	3.16	0.56	0.34
soybean meal	89	-	20.0	12.85	-	2.28
canola meal	93	-	5.0	11.8	-	0.54
corn gluten meal	90	-	6.0	2.78	-	0.15
total					0.92	3.6

Iron content

ingredient	level present		iron content mg/kg (fed basis)	iron content calculated (mg)	
	animal	plant		animal	plant
corn	57.8	47.3	21	1213.8	993.3
wheat	20.6	12.3	40	824	492
meat meal	6.0	-	440	2640	-
poultry byproducts meal	6.0	-	440	2640	-
fish meal	6.0	-	300	1800	-
feather meal	3.0	-	76	228	-
soybean meal	-	20.0	120	-	2400
canola meal	-	-	159	-	795
corn gluten meal	-	6.0	400	-	2400
T. B. mineral mixture	0.05	0.05	4.4(%)	2200	2200
poultry mineral mixture	0.05	0.05	3.0(%)	1500	1500
total				13045.8	10780.3

Appendix F

Composition of buffers

TAE (Tris acetate) buffer

Concentrated stock - 50X

242g Tris base
57.1mL glacial acetic acid
100mL 0.5 M EDTA (pH 8.0)

Working solution - 1X

TBE (Tris borate) buffer

Concentrated stock - 5X

54g Tris base
27.5g boric acid
20mL 0.5 M EDTA (pH 8.0)